MICROBIOLOGICAL REVIEWS, June 1994, p. 211–232
0146-0749/94/$04.00+0

Vol. 58, No. 2

The Bacterial Nucleoid Revisited

CARL ROBINOW[1]* AND EDUARD KELLENBERGER[2,3]*

Department of Microbiology and Immunology, Health Sciences Centre, University of Western Ontario, London, Canada N6A 5C1,[1] and Institut de Génétique et de Biologie Microbiennes, Université de Lausanne, CH-1005 Lausanne,[2] and Biozentrum der Universität Basel, CH-4056 Basel,[3] Switzerland

REFLECTIONS ON THE HISTORY OF BACTERIAL CARYOLOGY 211
GROSS ANATOMY OF THE NUCLEOID: RECALL AND REVISION 212
The Road from "Nuclear Material" to "Nucleoid" and Back Again 212
HCl-Giemsa Must Be Taken with a Grain of Salt 213
Acid Hydrolysis of Osmium-Fixed Bacteria Endows Nucleoids with an Affinity for Purple Components of Standard Giemsa Stain Which They Normally Lack 214
The Nucleoid in Bouin-Thionin Preparations 215
The Nucleoids in Cyanobacteria Remembered 216
ELECTRON MICROSCOPY OF BACTERIAL DNA-PLASMS IN SITU 216
Early Results of the Electron Microscopy of Bacteria and the Evolution of RK Fixation 216
Conciliation of Nucleoid Morphology as Seen in the Light Microscope with That of Nucleoids in Thin Sections 221
CHANGING VIEWS OF THE NUCLEOID 222
Shortcomings of RK Fixation, and How They Were Overcome by Autoradiography 222
New View of the Nucleoid Provided by CFS Treatment 223
FUNCTION-STRUCTURE RELATIONSHIPS 224
Tracing Sites of DNA and mRNA in Sections of CFS-Treated Bacteria 224
DNA 224
RNA 224
The Shape of Nucleoids Reflects the Level of Their Metabolic Activities 224
Ill-Defined Interlinked "Nucleoids" 225
Looking for a Resting DNA-Plasm 225
GIANT BACTERIA 225
A SIDEWAYS GLANCE AT THE CHROMOSOMES OF DINOFLAGELLATES 226
CURRENT CONCERNS 228
ACKNOWLEDGMENTS 229
REFERENCES 229

REFLECTIONS ON THE HISTORY OF BACTERIAL CARYOLOGY

Unbending after a deep bow in the direction of Leeuwenhoek and Pasteur, we may reasonably assert that modern bacteriology had its beginnings in the last quarter of the nineteenth century. The gross anatomy of bacterial cells is readily described in a few short paragraphs, and their component parts are well within the resolving power of microscopes equipped with the highly corrected objective lenses and compensating eyepieces that became generally available in the 1880s. In this essay we are trying to explain why, despite all this, it is only very recently that the nature of the bacterial nucleoid has been clearly revealed and why even now there are uncertainties about some of its features. Bacteriologists working in the newly created laboratories of hospitals and Public Health Authorities were first and foremost concerned with diagnosis. They were microscopists who looked, one might say, for, not at, bacteria. Efficient ways of looking for bacteria had been brilliantly demonstrated by Koch (68) with his photographs of *Bacillus anthracis* and other bacteria stained with synthetic dyes that had recently become available. Refined to the level of the Gram procedure and of special methods for certain hard-to-stain organisms, these diagnostic morphological techniques of the laboratory are still in daily use worldwide. By and large they reduce bacteria to a kind of Morse code of solidly colored dots and dashes (cocci and rods) lacking internal differentiation.

Such images did little to arouse cytological curiosity, and in the busy laboratories of applied bacteriology the study of bacterial cell structure was indeed rarely pursued. Two other factors contributed to the then prevailing lack of interest in bacterial cytology: the seemingly glass-like featureless translucency of living unstained bacteria from young cultures, and the failure of well-tried old, natural histological stains such as carmine and hematoxylin and a host of new synthetic ones to reveal significant intelligible structures within the kind of bacteria most often encountered in medical laboratories.

Microbiologists who had kept aloof from the "Hetzjagd der Bakterienstreberei" (approximately "the hot chase after notoriety in the field of bacteriology") grimly noted by deBary (18) continued to ask questions about the anatomy of the bacterial cell and were thus led to look for something corresponding to a nucleus. They seem to have been an industrious lot, to judge

* Corresponding authors. Mailing address for C. Robinow: Department of Microbiology and Immunology, Health Sciences Centre, University of Western Ontario, London, Ontario, Canada N6A 5C1. Phone: (519) 661-3427. Fax: (519) 661-3499.

Mailing address for E. Kellenberger: Institut de Génétique et de Biologie Microbiennes, Université de Lausanne, Rue César-Roux 19, CH-1005 Lausanne, Switzerland. Phone: 41 (21) 3206075. Fax: 41 (21) 3206078.

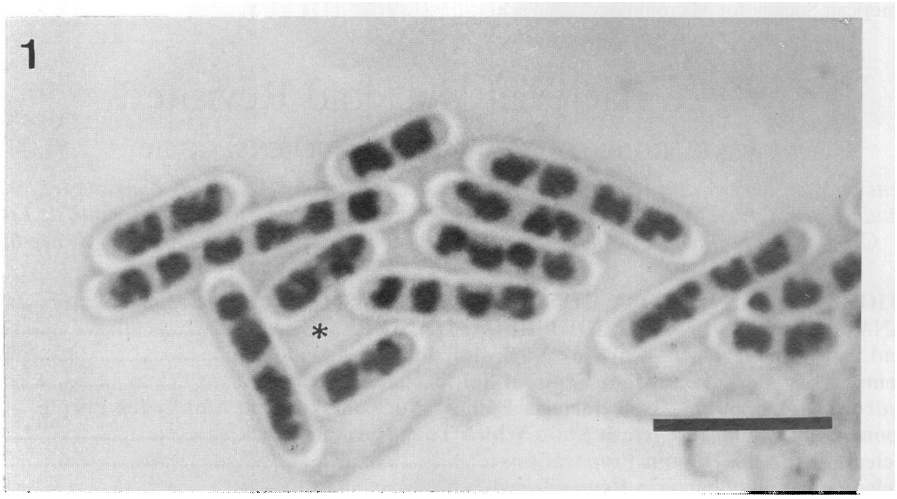


FIG. 1. Nucleoids in cells from a growing culture of a *Bacillus* species isolated from greenhouse soil, fixed with osmium tetroxide vapor and stained with HCl-Giemsa. The nucleoids of the three bacilli below, above, and to the right of the asterisk were preserved while going through the typical Piekarski round in which two nucleoids divide to make a set of four. Bar, 5 μm.

by the more than 400 papers on eubacterial and cyanobacterial cytology reviewed by Delaporte (21) in the introduction to the record of her own far-ranging observations. The bacterial cell, it could no longer be doubted, has its own kind of "corps chromatique," probably as necessary for the performance of vital functions and for reproduction as are the nuclei of the cells of higher organisms. However, the occurrence of bacterial "materiél nucléaire" in a protean array of configurations, including axial filaments, networks, and random scatters of irregular granules, suggested to the reviewer, not unreasonably, that the forms assumed by bacterial chromatin had no obvious functional significance. Large, promising species, occasionally brought in from the wild, tended to disappoint their discoverers because they often could not be propagated in the laboratory. Established ubiquitous species such as *B. mycoides* and *B. megaterium* were not neglected but tended to be examined at late stages of their growth cycle, when coordination of the reproduction of chromatinic elements with that of the organism containing them, obvious in cells from fast-growing young cultures, is often no longer apparent. It is easy to see why, up to the end of the 1930s, bacterial cytology had failed to attract the attention of caryologists, who saw more rewarding and challenging materials for the exercise of their skills in the large nuclei of the cells of higher organisms and the often enigmatic ones of protozoa.

GROSS ANATOMY OF THE NUCLEOID: RECALL AND REVISION

The Road from "Nuclear Material" to "Nucleoid" and Back Again

The introduction into cytology in the mid-1920s of the highly specific Feulgen procedure for the detection of sites of "thymonucleic acid" (alias DNA) allowed nuclear material in bacteria to be identified with enhanced confidence. Delaporte (21) regularly made use of the Feulgen procedure, as did several of the authors of the more recent papers of the large collection of studies reviewed by her. Most influential among these proved to be the work of Piekarski (87), which owed its success to the combination of several attractive features. His work was done on enteric bacteria that were well known and available in clinical laboratories everywhere. Most importantly, the bacteria were cultured under conditions which made them grow with short generation times. It was in samples of cells from such cultures that Piekarski found regular numbers of neatly spaced Feulgen-positive bodies (also seen by Stille [117] in cells from young cultures of *B. subtilis*) of a narrow range of sizes, whose orderly behavior suggested that they represented some kind of basic entity whose reproduction was correlated with that of the bacteria containing them and which the author appropriately, if somewhat boldly, named "nucleoids."

At the same time it was shown by Piekarski that the sites of the distinctly but only faintly positive Feulgen reaction could, after the acid hydrolysis which forms step I of the Feulgen procedure, be brightly and selectively colored with some of the components of the complex Giemsa stain (this protocol will be referred to in this review as HCl-Giemsa). Lastly, the results of this work were presented in unambiguous photomicrographs. Nucleoids were soon demonstrated in several other kinds of bacteria by Neumann (81), who had obtained good results with straight Giemsa stain. Further confirmation of Piekarski's and Neumann's work was provided by Robinow (90–93, 95), who favored the cells of young *Bacillus* cultures and relied chiefly on HCl-Giemsa (Fig. 1). Tulasne and Vendrely (119) reported the important observation that digestion with RNase was as effective as acid hydrolysis in making bacterial nucleoids selectively stainable with the Giemsa mixture. Lastly, the bright yellow-green DNA-specific fluorescence of acridine orange enabled Anderson et al. (1) to propose a plausible sequence of growth and division of nucleoids from observations of fixed preparations of *Escherichia coli*, a sequence that was in good agreement with what had meanwhile been learned about the behavior of nucleoids in the cells of living bacteria examined under the phase-contrast microscope by Knoell and Zapf (67), Stempen (116), and Mason and Powelson (73). Invariably the nucleoids of living, growing bacteria were found to stand out from the cytoplasm by virtue of their uniformly low density throughout cycles of growth and division, their soft irregular outlines, and their relatively large volume (Fig. 2). It was these experiences that provided the standards by which, later on, the quality of electron micrographs came to be judged. Thus, even before the isolated bacterial chromosome had been demonstrated by Cairns (13), the image of DNA-containing entities in bacteria had already changed from formless, anarchic material

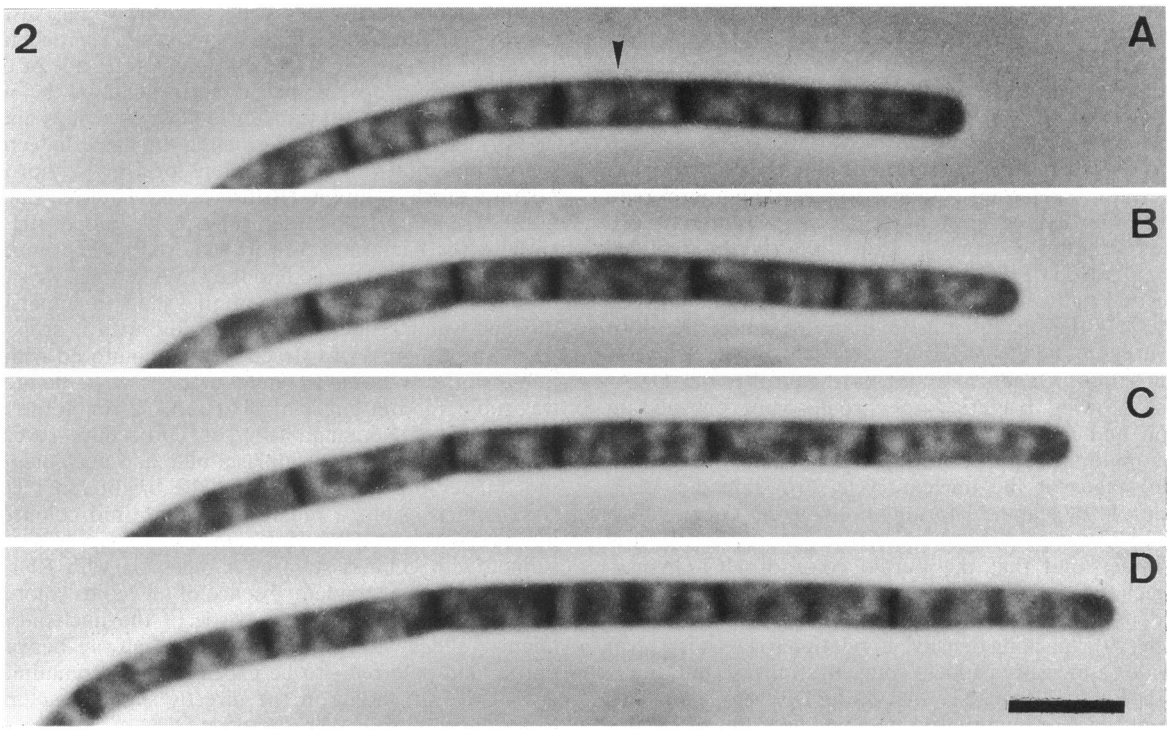


FIG. 2. Time-lapse phase-contrast photomicrographs, taken at 10-min intervals, of a chain of cells of a large bacillus ("*B. medusa*" [23]) growing in heart infusion agar with 16% gelatin. The four panels have been arranged so as to keep the third cell from the right in the center of the figure with no regard to relative displacements of points of reference. The increment in the length of the chain from panels A to D is therefore greater than it appears in this illustration. (A and B) The third cell from the right contains two nucleoids of complicated shape. (C and D) Division of the nucleoids has been accomplished. Bar, 5 μm. Reprinted from reference 95 with permission of the publishers.

to that of separately visible unit elements displaying orderly, predictable, and intelligible behavior. The findings of bacterial caryologists did not noticeably affect the course of bacterial genetics. Rather, the reverse is true; the molecular genetics of the bacterial genome made an honest organelle of the nucleoid only some 25 years after Piekarski (87) had first suspected its true significance. To turn to these early days, it cannot be denied that there developed a tendency to regard the tidily disposed ranks of translucent nucleoids in living bacteria and their Feulgen or HCl-Giemsa counterparts as the significant, functionally "correct" form of bacterial DNA-plasms, just as in earlier years the emphasis had been on their infinitely varied morphology. Electron microscopy was required to demonstrate the geometry-defying variety of shapes that bacterial DNA-plasms may assume and their ability to function equally well in the form of neat sets of (near) unit quantities or in the shape of irregular aggregates of unknown numbers of unit plasms. The two aspects are well illustrated by Fig. 2 of Woldringh and Nanninga (128), which shows a mixture of cells of the same strain of *E. coli* with generation times of 72 and 22 min. Nucleoids are clearly defined in the fast-growing cells, whereas those of the cells with long generation times are present as columns of granules lacking obvious regularity of behavior.

Therefore, the presence of a degree of plasticity of bacterial DNA-plasms unlike anything encountered in the nuclear behavior of eukaryotes should certainly be kept in mind; however, predictably ordered, functionally intelligible arrangements of material within cells will always appear more interesting to the inquisitive biologist than a random scattering. Thus, despite the shortcomings of osmium-HCl-Giemsa preparations (discussed below), images such as Fig. 1 still suggest that in cells from young, fast-growing *Bacillus* cultures the behavior of the nucleoids is both orderly and intelligible. Demonstrations of this kind may more effectively move a young microscopist to explore the behavior of DNA-plasms in growing, dividing bacteria than will many a textbook diagram of a single bacterial cell.

HCl-Giemsa Must Be Taken with a Grain of Salt

Two warnings must be sounded against taking OsO_4-HCl-Giemsa preparations at face value. First, the very neatness of the nucleoids (or, by another term, the confinement of the DNA-plasms) is actually due to a still not fully understood effect of the osmium fixation. Other morphological changes of the nucleoids, such as the very typical ones observed as consequences of various salt concentrations in the growth medium (56, 126), are caused by an osmium-induced loss of the selective pumping ability of the plasma membrane. Already suspected by these authors to occur in the first instances after a bacterium encounters straight osmium tetroxide, this loss was later proved to occur by Moncani (77; reported by Kellenberger [59]). Indeed, many substances, among them the commonly used aldehydes and osmium tetroxide, induce rapid leakage of potassium and influx of sodium. The loss of magnesium and calcium takes a little longer. During the last decades, aldehyde fixations (107) were increasingly favored by electron microscopists, who use it currently in a two-step procedure in which aldehydes are used before osmium. There is no place, however, for osmium in the very recently intro-

duced immunocytochemistry, since its presence prevents immune reactions from occurring.

To return to the bacteria, since osmium neither changes the number of nucleoids in a given cell nor significantly distorts the various stages of nucleoid growth and division, it cannot be said to generate misinformation about these matters. Such damage as is done is none too serious at the level of resolution attainable with the light microscope, and, used on appropriate samples, osmium-HCl-Giemsa has retained its usefulness as a technique for the ready demonstration of the "nucleoid mode" of the organisation of the bacterial DNA-plasm (Fig. 1).

The second matter to guard against when examining such preparations is the unconscious tendency to accept the deep reddish purple of the DNA-plasms as reflecting their character as chromosomes. Genetically and biochemically, the DNA-plasms of growing bacteria are chromosomes engaged in replication and transcription; however, as everyone knows, they differ materially from chromosomes of eukaryotes, which, at certain stages of the nuclear cycle, are stained a deep reddish purple by standard formulations of the Giemsa mixtures used without prior hydrolysis. To be more specific, it is vital to understand that the purple color of HCl-Giemsa-stained nucleoids is inconsistent with recent results about the packing density of their DNA. Introduced by Nanninga and Woldringh (79), "packing density" in relation to DNA is a term that provides a measure of local concentrations or, put differently, of mass per volume, e.g., that of the DNA in the head of a bacteriophage. For quantitative data and accounts of the use to which they have been put, see references 7 and 58. It appears that the packing density of the nucleoid of growing bacteria is only of the order of that of the interphase nuclei of liver parenchymal cells or hepatocytes. Therefore we can predict the shade that nucleoids ought to assume in Giemsa preparations.

Acid Hydrolysis of Osmium-Fixed Bacteria Endows Nucleoids with an Affinity for Purple Components of Standard Giemsa Stain Which They Normally Lack

Giemsa' reagent, as well as Wright's, Leishman's, and May-Grunwald's stains, are all members of the Romanowsky family of complex mixtures of eosin, methylene blue, and several oxidation products of the latter. Since their introduction about a century ago, these stains (or, rather, staining procedures) have been widely used by hematologists and parasitologists in the preparation of blood films for diagnostic scrutiny. Giemsa and Giemsa-like mixtures have also proved useful in histology. Giemsa colors interphase nuclei of tissue cells a bright transparent red with a tinge of purple, mitotic chromosomes deep shades of purple bordering on black, and cytoplasm sky blue. A discussion of the chemical information conveyed by this spectrum of color effects is beyond the scope of the present review, but convincing interpretations have been advanced by Jacobson and Webb (51). We conclude that, given the interphase-like low packing density of their DNA, the deep purple color of nucleoids in hydrolyzed preparations stained with standard HCl-Giemsa is inappropriate. If so, how is it acquired? It could be argued that nucleoids, especially those of growing cells, are normally stained purple by Giemsa stain but that they tend to be obscured by the large amount of stain accepted by the bacterial cytoplasm. With this view, if staining is preceded by acid hydrolysis, RNA is removed from the cytoplasm and subsequent staining with Giemsa gives the nucleoids a chance to be seen. Well, "It ain't necessarily so!" We arrived at this conclusion while looking for nucleoids during the slow removal of stain from the cytoplasm of bacteria stained directly with Giemsa stain. In this enterprise we used young, fast-growing cultures of four kinds of bacilli as well as *E. coli* on agar media, preserved in situ with vapors of osmium tetroxide or the fumes above a few drops of glacial acetic acid in the well of a depression slide. After transfer to glass coverslips and postfixation (and storage) in 70% ethanol, the bacteria were stained for various times in 1:10 dilutions of Gurr's improved R66 Giemsa stock solution (British Drug Houses Ltd., Poole, United Kingdom) with M/15 phosphate buffer (pH 6.8). Invariably the bacteria were soon colored a deep impenetrable bluish purple all over. To find the DNA-plasms, we carried out a slow, stepwise extraction of the stain in a petri dish of distilled water rendered slightly acidic with a drop of acetic acid. The progress of extraction was monitored with a water immersion lens during frequent intervals when the preparation was submerged in buffer at pH 6.8. Confirming the extensive experience of Neumann (81), our procedure revealed that straight Giemsa stains cytoplasm blue and nucleoids red. The red, in our experience, was not the bright red recorded by Neumann (81) but a red recalling the "red colouration" of nucleoids observed by Hartman and Payne (37) in *E. coli* stained with Giemsa stain after digestion with RNase. Most obvious was the complete absence of nucleoids colored in that deep reddish purple characteristic of the nucleoids in HCl-Giemsa slides. Moreover, nucleoids halfway between deep purple and pale red—to be expected if "chromatinic" nucleoids had been hiding in the directly stained bacteria—were never encountered.

We infer from these observations that, apart from achieving a welcome reduction of the basophilia of the cytoplasm, HCl acts directly on the nucleoids in a way which endows them with an affinity for purple components of the standard formulation of Giemsa's stain (whatever, precisely, that may be), which they normally lack. Our argument would gain strength if there were microorganisms in which both the cytoplasm and nucleoids are normally barely stainable by Giemsa stain but the nucleoids display a selective affinity for that stain after the organisms have been treated with warm N/1 hydrochloric acid. Such (honorary) organisms are found among mitochondria, now widely believed to be descendants of bacteria and known to harbor a prokaryotic type of DNA (3). Comparison of Fig. 3A and B reveals that the body and the nucleoids of mitochondria of the slime mould *Physarum polycephalum* are only faintly stained by straight Giemsa stain but that the nucleoids, as expected, acquire a strong affinity for purple components of that stain in the course of acid hydrolysis. The unusual strength of the coloring of the nucleoids is due to the high level of polyteny of the mitochondrial tDNA, estimated at 32 by Kuroiwa (69). It was, incidentally, not only the nucleoids of the mitochondria that responded in this way to hydrolysis; the affinity for Giemsa stain of the proper nuclei of *P. polycephalum* (but not, of course, of the nucleoli) was also greatly enhanced. This did not come as a surprise. We have long been aware of the usefulness of HCl-Giemsa in the study of nuclear behavior in fungi. The chromatin of interphase nuclei as well as that of dividing ones in vegetative structures of yeasts and molds, although Feulgen positive and fluorescing vividly in the presence of 4′,6-diamidino-2-phenylindole (DAPI), is, as a rule, lacking in affinity for common nuclear stains used in ordinary ways (83). However, like bacterial DNA-plasms, the chromatin of budding yeasts, hyphal nuclei, and conidiospores acquires a strong affinity for purple components of the Giemsa stain in the course of hydrolysis with N/1 HCl (94, 98–100). We are thus dealing with a puzzling aspect of certain chromatins with wider significance than the now rarely performed HCl-Giemsa staining of bacterial nucleoids.

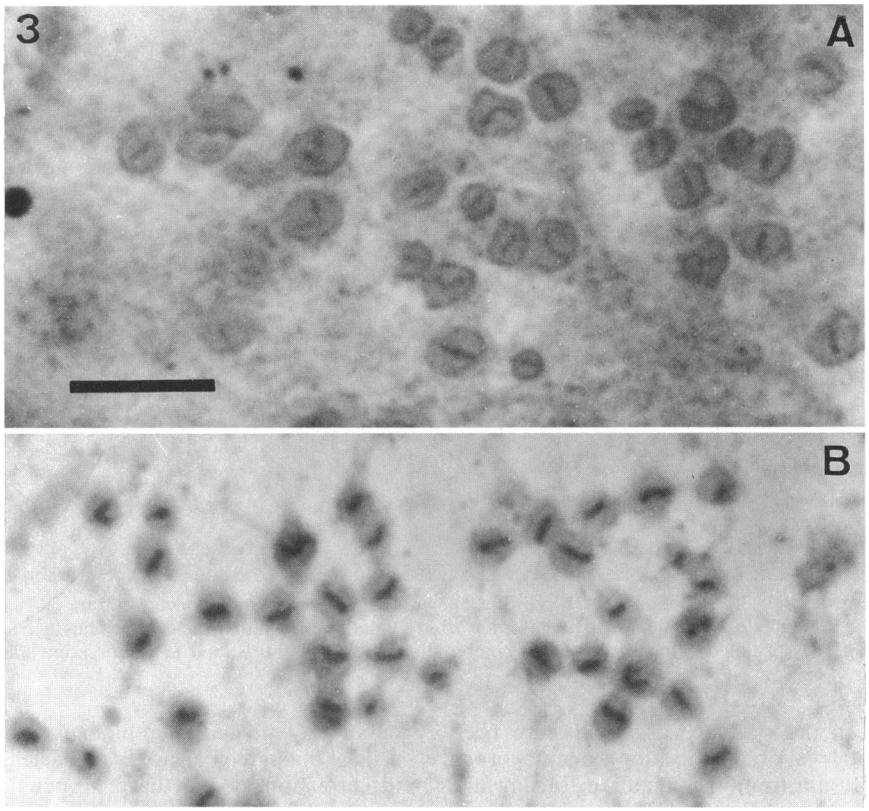


FIG. 3. Mitochondria of the slime mold *P. polycephalum* in drops of a plasmodium fixed in Helly's mixture plus 6% formalin. Both panels A and B were stained with Giemsa stain. (A) The nucleoids of the mitochondria are only just visible; the matrix of the mitochondria, too, is only faintly stained. (B) This sample was treated for 10 min with N/1 HCl at 45°C before being stained with Giemsa stain. The nucleoids now appear deeply stained. The visibility of their extracted matrix has been enhanced by brief staining with dilute basic fuchsin. The mitochondria are really short rods (69). Their rounded shape in this photomicrograph is an artifact of fixation. The shapes of their nucleoids, however, correspond to those seen in electron micrographs of sections of fixed pieces of plasmodia, kindly prepared for us by Robert Buck, University of Western Ontario. Bar, 5 μm.

Regarding the latter, it would be tempting but unwise to assume that the well-known loss of adenine and guanine from DNA during acid hydrolysis must be responsible for the enhanced affinity of the nucleoids of hydrolyzed bacteria for certain components of the Giemsa mixture. It would be unwise because a selective reddish-purple staining of bacterial nucleoids is equally well achieved by two modifications of the standard Giemsa procedure, those of Badian (4) and Piéchaud (86), which do not involve a prior treatment with HCl. Both modifications involve extra eosin. Badian used it to decolorize and differentiate stained preparations; Piéchaud recommends its use as a diluent additive to commercial Giemsa stain. The formulation of Piéchaud has been used to good effect by Delaporte (22). The two cases before us could possibly be explained by accepting that one of the functions of eosin in Giemsa stain is that of a mordant. However, as Baker (5), writing about Romanowsky stains, has aptly remarked, "In staining as in other branches of technique, practice often long precedes theory." We shall leave it at that. Indeed, terms such as Feulgen procedure and HCl-Giemsa have an antiquated ring in the age of DAPI (45, 127). We readily concede the distinctness of images provided by that fluorochrome (Fig. 4) but venture to suggest that its advantages are more apparent to cytochemists than to morphologists.

It remains to discuss the results of Bouin fixation followed by direct staining with thionine, a procedure for the light microscopy of bacterial nucleoids that is at once the most appealing of methods morphologically and (at present) the most frustrating one cytochemically.

The Nucleoid in Bouin-Thionin Preparations

When blocks measuring, say, 2 by 5 mm, are cut out of growing monolayer cultures of bacteria on an agar medium, placed cells down on coverslips, and immersed in Bouin's fixative (as first demonstrated to C.R. by the late Emmy Klieneberger-Nobel of *Mycoplasma* fame), some hours later the bacteria will have been transferred to the glass slips more or less quantitatively and with little dislocation (93, 95, 101). Alternatively, bacilli may be grown from spores dried on coverslips, covered with thin narrow strips of agar, and kept in a moist chamber until fixation of the new growth in situ by percolation of Bouin's reagent through the agar. Brief staining with 0.003% thionin in tap water or with low concentrations of other dyes accepted by the cytoplasm reveals the nucleoids in the form of distinct species-specific "silhouettes" which bear a close likeness to the dimensions and changing configurations of the nucleoids of living, growing bacteria examined by phase-contrast microscopy. However, no chromatin is revealed at the sites of the nucleoids of Bouin-fixed bacteria by either the Feulgen or HCl-Giemsa procedure. Furthermore, the first results of fluorescence microscopy of Bouin preparations of *B.*

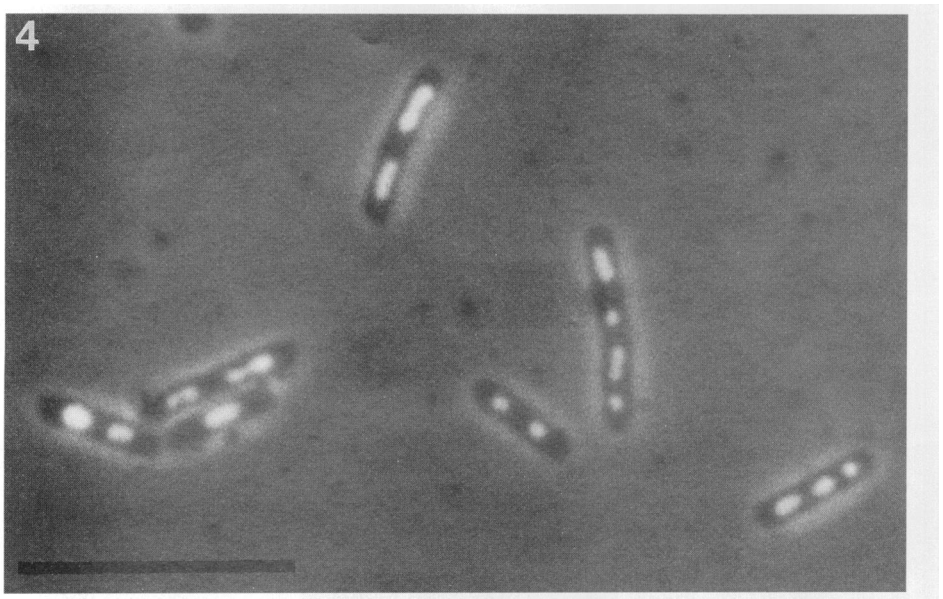


FIG. 4. *B. subtilis* 168 grown in broth to about 5×10^7 cells per ml, with 2 mg of DAPI added to the culture 30 to 60 min before being placed between a coverglass and a thin layer of dried gelatin. The latter absorbs the medium, such that the cells are finally lying in a single plane and still in growing conditions (63). The specimen is then observed by combination of phase-contrast and UV fluorescence. Bar, 8 μm.

subtilis 168 examined in the presence of DAPI have been ambiguous. Experimental work on the fixation dynamics of the valuable but complex Bouin fixation is required. Meanwhile, we are encouraged by the good overall correspondence between the pattern of DAPI-stained nucleoids in glutaraldehyde-fixed *B. mycoides* (Fig. 4) and their counterparts in the form of translucent silhouettes in Bouin-thionine preparations of the same species (Fig. 5). We are equally pleased to note good correspondence between the complex shapes of the nucleoids in our Bouin preparations of *B. megaterium* (see Fig. 7) and of nucleoids demonstrated by DAPI fluorescence in glutaraldehyde-fixed *B. megaterium* cells by Setlow et al. (111). At the same time we are obliged to point out that the evidence of Bouin-thionine and, more obviously, osmium-HCl-Giemsa preparations (Fig. 6) clearly shows that growing cells of *B. megaterium*, like those of other large and small members of the genus *Bacillus*, contain two, three, or four separate nucleoids in more or less advanced states of replication, not just one long lobulated nucleoid extending over the whole length of a bacillus as maintained by the authors. Four separate U-shaped (i.e., dividing) nucleoids are, in fact, plainly visible in the two arrowed bacilli of Fig. 2c of Setlow et al. (111).

Figure 7A shows that growing cells of *B. megaterium* harbor several separate nucleoids; Fig. 7B illustrates the habit of this bacillus of growing in the form of chains of paired dividing cells.

One familiar with Bouin-thionine preparations can have little doubt that the orderly arrays of "vacuoles" in living, unstained cells of "*B. oxalaticus*" (probably *B. cereus*) of which Migula (75) provided a photomicrograph were really the sites of nucleoids.

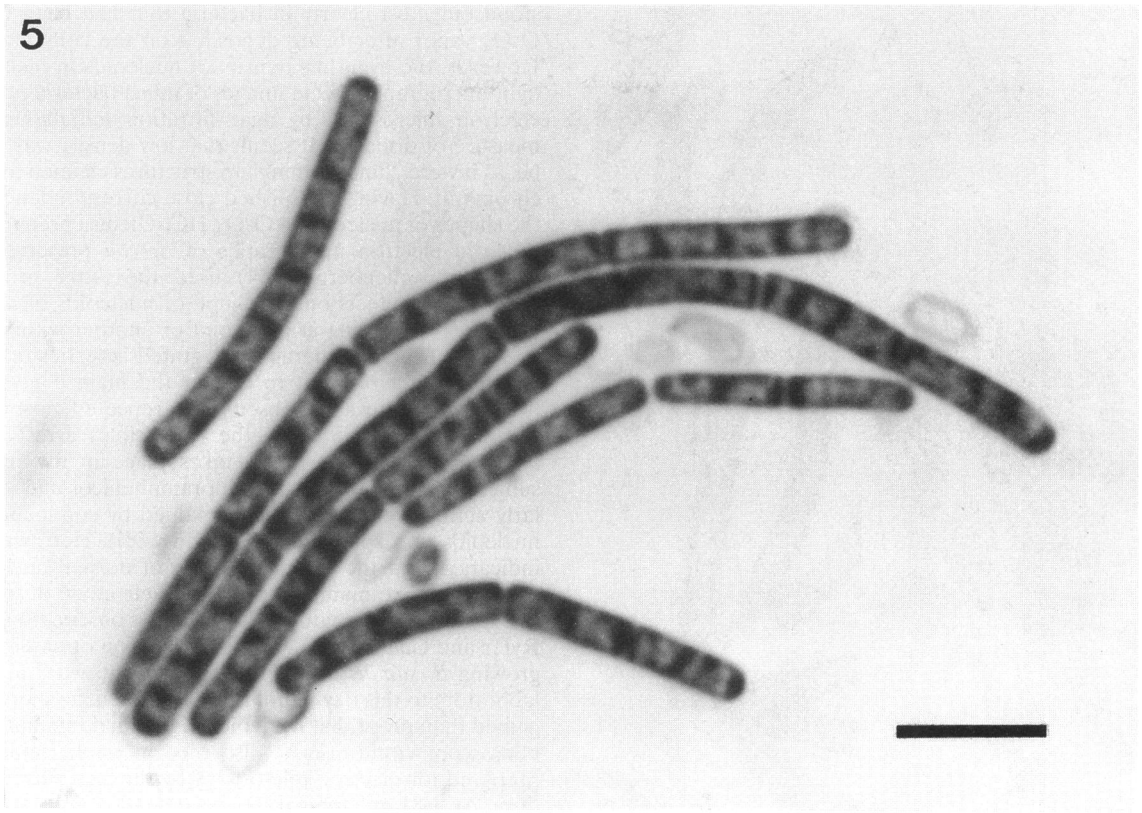
The Nucleoids in Cyanobacteria Remembered

Our account so far has dealt chiefly with the configuration and behavior of the chromatin in fast-growing eubacteria as revealed by certain forms of light and electron microscopy. It would be absurd to fail to mention at this point that, long before bacterial nucleoids began to be seriously studied, botanically minded microbiologists had already seen that the cells of blue-green algae (cyanobacteria), although not lacking in chromatin, do not have proper nuclei. Volume 2 of the monumental treatise on the structure and reproduction of the algae by Fritsch (28) devotes several pages to the enigmatic chromatin complexes of blue-green algae, reproduces many telling illustrations of them from the older literature, and draws attention to the positive Feulgen test of their centroplasm, as demonstrated, among others, by Delaporte (21). Fritsch declared that "... any comparison of these structures with the nuclei of other algae is impossible." Let us say that when such a comparison is attempted, one finds that cyanobacterial chromatin, to judge by its direct mode of division and lack of an envelope, is clearly that of prokaryotic organisms. Excellent photomicrographs of stained cyanobacterial nucleoids are given in a paper by Cassel and Hutchinson (15). Electron micrographs of sections of cyanobacteria preserved by the methods of Ryter and Kellenberger (108), published in a monograph by Fuhs (30), show their chromatin to be structured just like that of ordinary bacteria preserved in the same manner. Lastly, Fig. 8, a photomicrograph of a *Cylindrospermum* species, makes the same point once again with the help of light microscopy.

ELECTRON MICROSCOPY OF BACTERIAL DNA-PLASMS IN SITU

Early Results of the Electron Microscopy of Bacteria and the Evolution of RK Fixation

When electron microscopes became widely available in the late 1940s, it was expected that they would soon provide more detailed information about the organization of nucleoids than was within reach of the light microscope. These expectations were not immediately fulfilled. In early trials, bacteria, especially *Bacillus* cells, appeared equally dense all over, with the degree of density varying from black to gray according to whether low or high voltages had been used. It is true that Knaysi and Baker (66) noted numerous "vacuoles" in intact



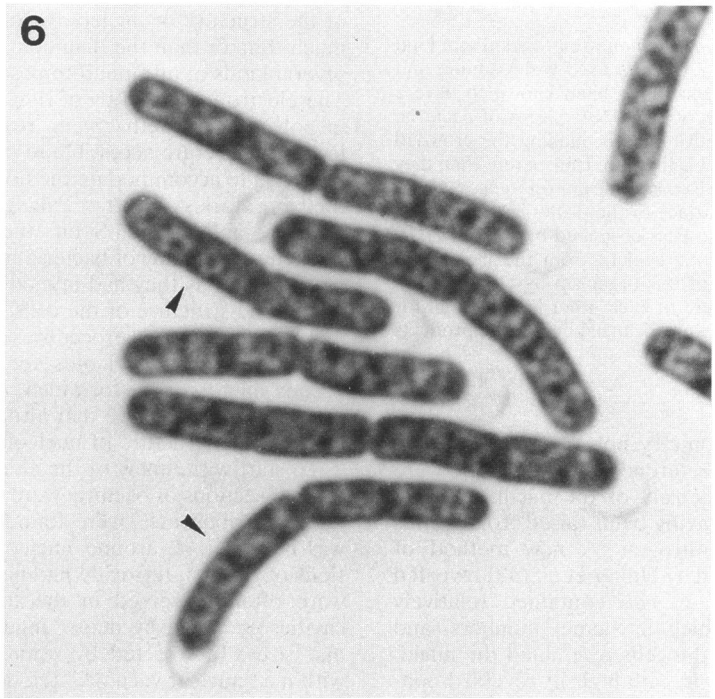


FIG. 5. A microcolony of *B. cereus* subsp. *mycoides* grown in a moist chamber from spores that had been dried on a coverslip, covered with a narrow sliver of thinly poured agar, and fixed in situ by percolation of Bouin's reagent through the agar. Staining was with 0.003% thionin for 80 s. The rinsed coverslip was then mounted over a drop of water and photographed in the orange light transmitted by Kodak Wratten filter no. 22. Most of the cells of the third chain from the top are in best focus. Nucleoids, represented by their translucent silhouettes, are seen in all stages of growth and division. Their configurations, with a few "wild" ones among ordinary nucleoid shapes, are characteristic of *B. mycoides* and reliably distinguish it from the related *B. cereus*. Bar, 5 μm.

FIG. 6. *B. megaterium*. The pattern created by the nucleoids of the huge bacilli may, at first sight, resemble some cryptic ancient inscription but is essentially the same as that of the arrangement of the nucleoids in Fig. 5. Thus, the arrowed bacilli each contain four dividing nucleoids. Elsewhere, division of the nucleoids has evidently not been entirely synchronous. Magnification is nearly the same as in Fig. 5.

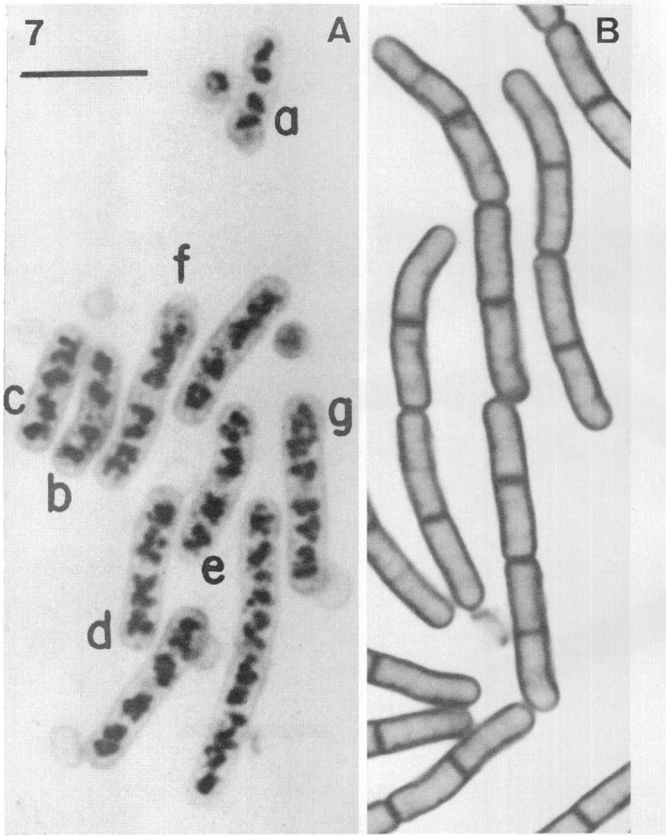


FIG. 7. These illustrations of *B. megaterium* are provided as a kind of Rosetta stone to explain Fig. 6. (A) Cells fixed with osmium and stained with HCl-Giemsa. Osmium has long been known to have a confining effect on the bulk portion of the DNA-plasms of bacteria. Here, this effect is turned to our advantage in making the crowded patterns of nucleoids in the growing bacilli more transparent than they are after Bouin fixation. Bar, 5 μm. Reprinted from reference 93 with permission. (B) The cell wall and surface of the protoplast have been selectively stained with Victoria blue B as described by Robinow and Murray (101). In *B. megaterium*, as is evident from the illustration accompanying the first description of this organism by DeBary (18), recently divided bacilli tend to adhere to each other long enough for the next round of cell division to be well advanced before the products of the previous division finally separate.

cells of *B. mycoides*, whose geometry now strongly suggests, most obviously in Fig. 3b of reference 66, that they were nucleoids. The unusually low density of the bacilli in these preparations was due to their having been raised from spores in a medium lacking added nitrogen. A new method of specimen preparation introduced by Hillier et al. (42) revealed that normal, growing cells of *E. coli* contained relatively electron-translucent regions which in shape, numbers, and manner of arrangement within the cells resembled the nucleoids of HCl-Giemsa preparations, although in reversed contrast. There was also encouraging correspondence with phase-contrast light micrographs published by Stempen (116), which showed the nucleoids of growing *E. coli* and *Proteus vulgaris* cells to be less dense than their cytoplasm. Informative electron micrographs of intact whole cells of *E. coli* were next obtained from specimens obtained by improved methods of "agar filtration" (53, 54, 62). In this procedure, bacteria are deposited on collodion by filtration through a thin film of this electron-lucent material atop an agar medium. Nucleoids stood out most clearly in bacteria that had been exposed to OsO_4 vapor after being deposited on the collodion (Fig. 9). However, the even low density of nucleoids in phase-contrast light micrographs and in images of intact bacteria prepared for electron microscopy by agar filtration initially met with a measure of disbelief. It seems that low density was thought to be, as it were, "unbecoming" to structures claimed to represent chromatin. Having established close correspondence between the shapes of nucleoids in OsO_4-HCl-Giemsa preparations and those in electron micrographs of *E. coli* preserved by agar filtration, Kellenberger (54) used the latter procedure to examine the effects on the shape of nucleoids of a variety of measures interfering at one point or another with the normal working of the innumerable integrated activities of the bacterial cell. Most relevant to one of the main concerns of the present account was his observation, repeated most recently by Bohrmann et al. (8), that the (reversible) arrest of protein synthesis that, for instance, takes place in the presence of sublethal concentrations of chloramphenicol and other similarly acting antibiotics is accompanied by confinement of the nucleoids into spherical shapes (Fig. 9B). Here was an early indication that the extended shape of normal nucleoids was, perhaps, in like manner, reflecting their mode of functioning; this belief was strengthened in time by observations made by Ryter and Chang (104) on the distribution of nascent RNA in growing *B. subtilis* and has influenced the work at the Basel laboratory to this day. Until this point, then, no more had been gained than proof that nucleoids of the kind familiar from light microscopy could also be discerned in whole, intact bacteria prepared for electron microscopy by the agar filtration procedure. At the same time, it had become obvious that any details of the structure of nucleoids would be revealed only in slices much thinner than the diameter of bacteria. In the late 1950s, several kinds of ultramicrotomes had become widely available. The electron microscopy of thin sections of tissues embedded in polymerized methacrylate resins had begun to make new levels of structure accessible to cytologists. New journals were launched to accommodate the flood of reports emanating from the laboratories of Porter, Palade, Sjoestrand, Bernhard, Irene Manton, and others. Soon, the new techniques were also applied to the study of bacteria in the hope that they would be as rewarding as they had immediately been in the exploration of the fine structure of the cells of higher organisms. Surprisingly, the search for procedures for the satisfactory preservation of bacterial nucleoids for electron microscopy would occupy microscopists for much of the next 30 years, and even now some of us believe that ultimate certainty about the form and the fine structure of nucleoids still escapes us.

To start with, many of the electron micrographs of the first crop of sections of bacteria were disappointing. No organelles, not even ribosomes, were found in the cytoplasm, and there was no envelope around nucleoids. Worse, even when solutions of osmium tetroxide had been used as fixative, nucleoids were often preserved in the form of bizarre electrolucent cavities occupied by dense angular bodies of various shapes and sizes. Interpreted by some as images of chromosomes within a "nuclear vacuole," the dense bodies were regarded by others merely as the products of poor fixation. The choice of a suitable fixative was, however, not the only problem facing bacterial cytologists. Some resin-embedded bacteria, in addition to containing the aforementioned black bodies, appeared swollen, whereas others seemed to have been explosively disrupted. Since distortions of this kind were never seen to occur during fixation or dehydration, it was concluded that they must arise during polymerization of the methacrylate resins chosen as the embedding medium (9). Less accident-provoking

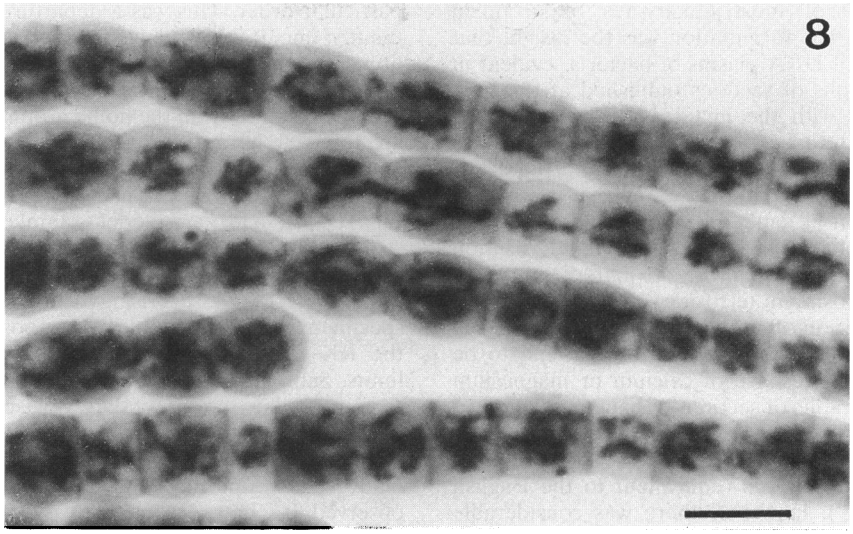


FIG. 8. Trichomes of a *Cylindrospermum* species from the late R. Y. Stanier's collection of pure cultures of cyanobacteria at the Institut Pasteur, Paris, France. The cells are stained with osmium vapour, Helly, and HCl-Giemsa. Bar, 5 μm.

resins were looked for and found in the epoxies and polyesters which soon replaced methacrylates (36, 65, 105). This change of embedding media brought an end to swelling and explosions but left unsolved the problem of aggregation, i.e., the creation of opaque chunks or ribbons of dubious nature floating in the "nuclear vacuole." A systematic study of fixatives, able to protect the fabric of nucleoids from aggregation during preparation for electron microscopy, was therefore undertaken by Ryter, Kellenberger, and their colleagues at Geneva (57, 106), who aimed at the development of a fixation method that would preserve bacterial DNA-plasms in something like the finely structured condition of nuclear material in certain electron micrographs of sections of bacteria then newly published by Chapman and Hillier (16). The Ryter-Kellenberger (RK) or standard fixation procedure finally developed is no longer considered optimal by its designers but is still worth discussing because it led to the discovery of the high aggregation sensitivity of the chromatin of prokaryotes, a character trait not

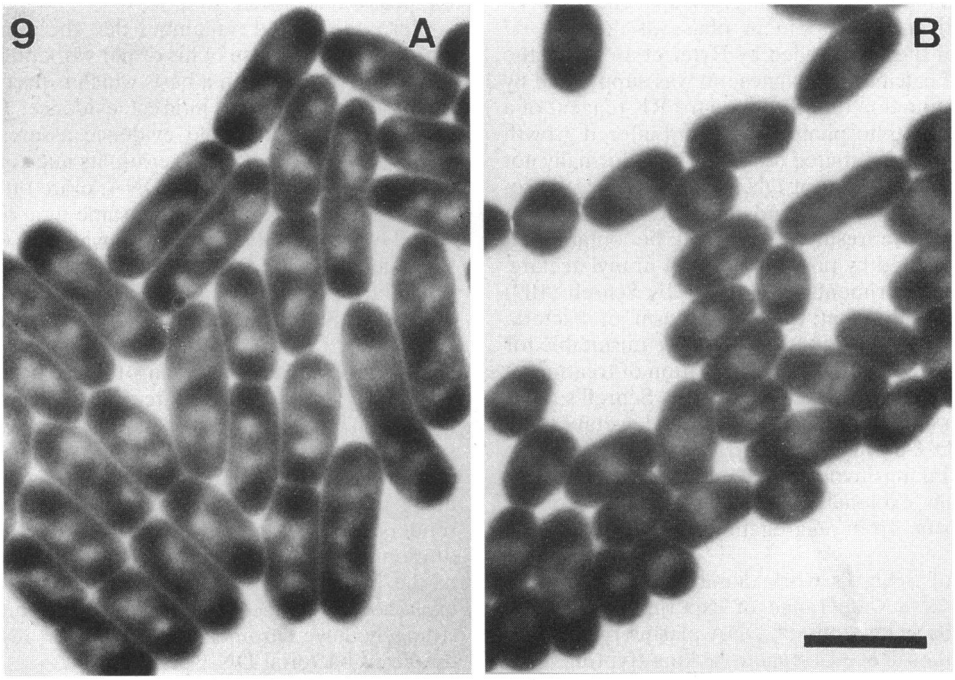


FIG. 9. *E. coli* prepared by "agar filtration" for global electron microscopy. After the cells are deposited on collodion-coated agar, they are fixed in osmium tetroxide vapor; the film is then floated off the agar by oblique immersion of cut-out blocks of the coated agar in distilled water. (A) *E. coli* K-12 cells from an exponentially growing culture. (B) The same strain of *E. coli* after treatment with a sublethal dose of an inhibitor of protein synthesis. Bar, 1.5 μm.

shared by the chromatin of (most) eukaryotes. Important in the development of the RK formulation was the insight that the coarse aggregation of DNA-plasms of bacteria, evident in most electron micrographs of sections published at the time and so greatly at odds with the images of evenly textured nucleoids provided by the microscopy of living bacteria, was not the result of fixation with osmium but occurred later, during the dehydration by ethanol or acetone that is required before fixed samples can be embedded in resins. It was this special vulnerability of bacterial chromatin that RK fixation was designed to overcome. The principal ingredient of the fixative continued to be osmium tetroxide, but for reasons not yet understood, the addition of a mixture of amino acids (e.g., Bacto-Tryptone or Difco Casamino Acids) was found to be critical. So, too, was the presence of calcium or magnesium ions. In electron micrographs of sections of bacteria preserved by this RK formulation, DNA-plasms appeared, at last, as loose networks composed of strands of various thicknesses rated "assez fins" by the authors (equivalent to the English "fair" or "not too bad"). However, there was considerable further improvement when 16 h in RK fixative was followed by 2 h of separate, secondary fixation with uranyl acetate, whose persistent low pH of about 3, even in buffered solutions, precluded its direct addition to the RK reagent. Thus RK became RK→U. By using a minimal effective concentration of uranyl acetate, Séchaud and Kellenberger (110) were eventually able to combine this important reagent with aldehyde in a one-step fixative which gave good results. The two-step RK→U procedure regularly produced DNA-plasm having the appearance of a web or tissue of fine fibrils (rated "très fin") of uniform thickness and usually lacking distinct orientation (Fig. 10A). In many laboratories it is this protocol that, to the chagrin of its designers, is often still referred to as RK fixation, with the role of postfixation with uranyl acetate being demoted to that of a mere staining procedure.

The ability of uranyl acetate to provide protection is well illustrated by the analysis of various mishaps encountered during the research and development phase of the RK→U system. In several of these, recorded by Ryter et al. (106), the beneficent action of calcium or magnesium was suppressed by the unintended introduction into the standard RK reagent of a chelating agent such as phosphate ions from buffered growth media. Fixation with this mutilated reagent would normally not have prevented the aggregation of DNA-plasms during subsequent dehydration, but it was found that even under these circumstances acceptable results could still be obtained if dehydration was preceded by postfixation with uranyl acetate. Further and similar experiments carried out by Schreil (109) and Fuhs (29) confirmed that the DNA-plasm of bacteria (imperfectly) preserved by fixations known to be unsuitable for the purpose could be rescued by the intervention of treatments with uranyl acetate. Of particular interest was Schreil's demonstration (109), on a test tube scale, that uranyl acetate turns solutions of pure DNA into stable, birefringent gels. Here, then, in the cross-linking involved in creating this effect, we may see a probable explanation of how RK→U protects bacterial DNA-plasms from aggregation by organic dehydrants.

Another aspect of Schreil's work demands our attention. During the course of a wide range of experiments, he had noted that the fibrils in sections of DNA-plasms of bacteria preserved in a modified RK→U reagent lacking tryptone had, here and there, formed "distinct ordered arrangements." This finding represents an important departure from the prevailing standard of the RK→U pattern of DNA-plasms composed of a sponge- or feltwork of fine fibrils not arranged in any particular order. This was a departure indeed, but it was not the first one. It is instructive to find a distinct ordering of DNA fibrils in most of the densely textured nucleoids in sections of one of the first lots of bacteria preserved with RK→U reagent at some distance from the home ground of that procedure. We are referring here to the beautiful micrograph of a section of *Salmonella typhimurium* contributed as Fig. 8 by Birch-Anderson and Maaloe to Ryter and Kellenberger (106), whose own Fig. 1D, 3, 4, and 7 are model examples of finely and randomly structured DNA-plasms. That this deviating result, duly but gently commented on by the principal authors, was obtained is, in retrospect, not surprising. Details of the preparation of specimens for fixation and the composition of the RK part of the RK→U procedure had been prescribed within narrow limits. Sampling the relevant literature of the 1960s, with its numerous illustrations of DNA-plasms with highly ordered texture, suggests, as Schreil (109) and Fuhs (29) had already conjectured, that the RK→U conditions of fixation had then, inadvertently and in good faith, not always been strictly observed. It seems reasonable to assume that slight imbalances of the ingredients of RK reagent, as well as variations in the length of exposure to that reagent, may have permitted the transformation of the normally supercoiled DNA into liquid crystals (see below), which were subsequently fixed by uranyl acetate in step II of the RK→U procedure. Such a course of events, we now believe, would account for the appearance in the literature of the 1960s of numerous electron micrographs of sections of nucleoids with their constituent fibrils aligned in parallel or in the form of whorls, arcs, or twisted ropes (see, e.g., references 26 and 124), including examples from the groups around Ryter and Kellenberger (56, 64). It undoubtedly added to the credibility of the results of the RK→U procedure that the fibrils of the DNA-plasm had diameters between 3 and 6 nm, well within the limits of accepted diameters of DNA molecules. Appealing and eminently plausible, these persuasively structured arrangements were often accepted as reflecting preformed order.

Here one should remember that the microscopist trying to assess the significance of his or her work on bacterial nucleoids must form opinions on a basis which is part hard fact and part inference drawn from indirect evidence. On the one hand, introconvertible manifold evidence assures the microscopist that during the life of a bacterium its nucleoid harbors a single, long, unbroken molecule of DNA, more often than not one in a state of replication and at the same time obliged to be tightly coiled on itself in the little space available. On the other hand, the microscopist cannot be unaware that chemical fixation procedures to which this long folded molecule are subjected are likely to induce departures from its natural, in situ conformation.

At this time, and returning to our proper subject, let us merely say that uranyl acetate in the RK→U reagent has two effects on DNA-plasms. First, by cross-linking neighboring strands of their DNA, it compensates for the low protein content of bacterial chromatin, which is the reason for its not being fixed by conventional fixatives. Uranyl acetate fixation renders the DNA-plasms resistant to aggregation during dehydration, but at the same time it also affects certain fine details of the fabric of DNA-plasms in roundabout ways. These are explained in the section dealing with dinoflagellates, which, strangely, have chromosomes that often resemble uranyl acetate-fixed bacterial DNA-plasms.

All in all, RK→U was a good fixative which achieved what its designers had hoped for. At the same time, as we shall see below, it did not make all preserved structures equally clearly visible.

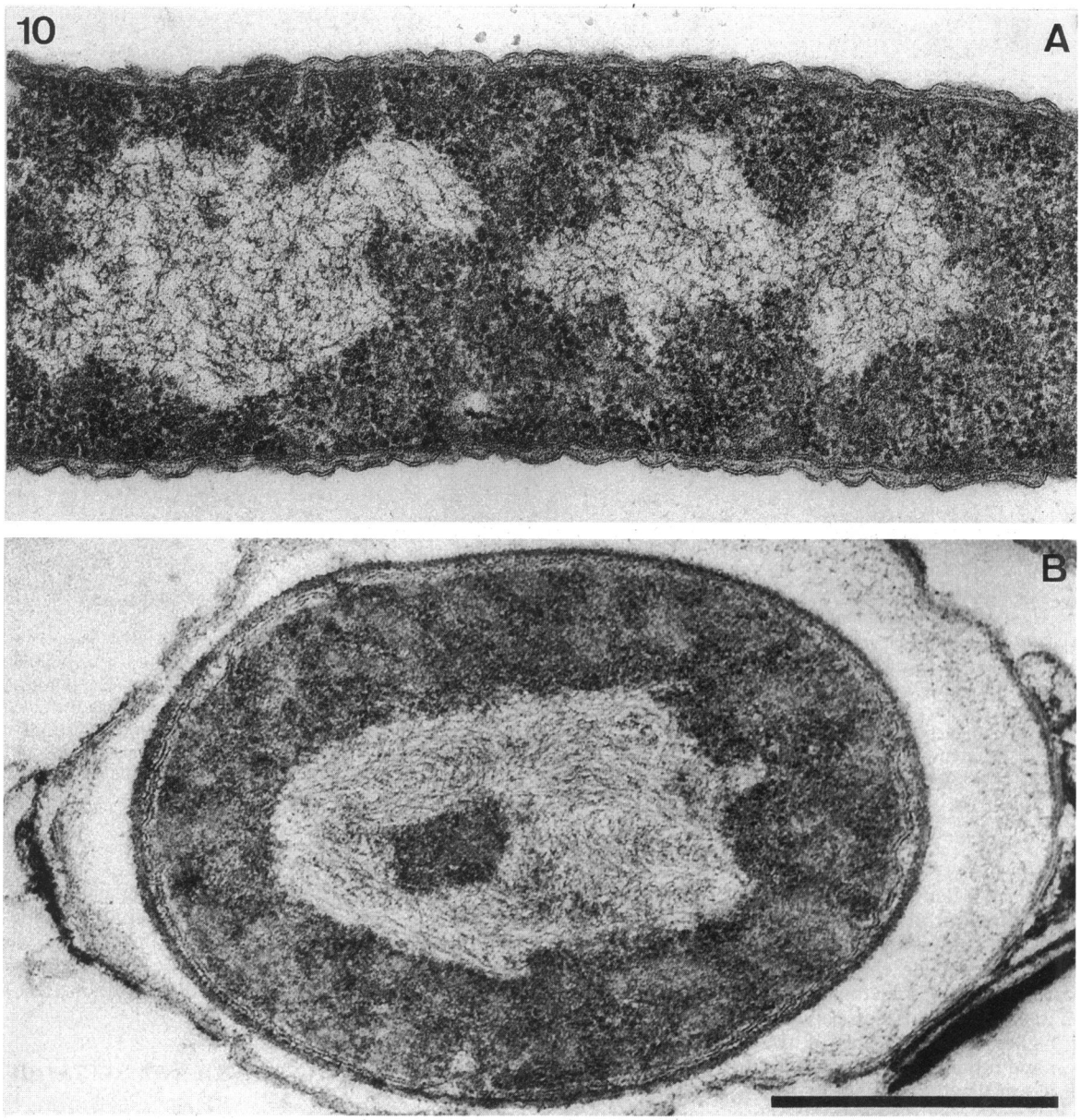


FIG. 10. Thin sections of cells that were fixed with osmium tetroxide and postfixed with aquous uranyl acetate. (A) *E. coli* cell with the network type of the DNA-plasm, as it is usually observed when the RK conditions are completely fulfilled. (B) Germinating spore of *B. megaterium*. The DNA-plasm is organized in the form of parallel bundles forming layered "whorls" comparable to those of the dinoflagellate chromosomes in Fig. 16A. Comparable aspects of the DNA-plasm are obtained when the cytoplasm is fixed with aldehydes and only later is DNA fixation in uranyl acetate (postfixation) performed. For more detail, see the text and Fig. 12. This micrograph is also noteworthy because it shows groups of resolved ribosomes within a relatively opaque ground plasma. Compare also the micrographs in Fig. 8 and the schematic drawing in Fig. 1 of Kellenberger and Ryter (64). Ribosome-free spaces are thus definable even for osmium fixation and not only after aldehyde fixation or cryofixation (Fig. 11 and 14). For further discussion, see the text. Bar, 0.5 μm. Panel B was prepared by John Marak, University of Western Ontario, around 1960.

Conciliation of Nucleoid Morphology as Seen in the Light Microscope with That of Nucleoids in Thin Sections

Let us pause here and consider what had and had not been achieved up to this time in nucleoid research. It should be emphasized that none of the cohort of early students of bacterial nucleoids, from Piekarski to Kellenberger, used the terminology of the mitotic cycle of the nuclei of the cells of higher organisms in writing about the subject of their studies; the exception, for several years, was DeLamater (20). Brock (11) has drawn attention to the one occasion when, referring a certain arrangement of bacterial chromatin bodies, Robinow (92) referred to telophase. He omits to mention that in the original context the offending word was placed in quotation marks, indicating that it was used there facetiously.

Asked to survey and interpret current work on the chromatin bodies of bacteria, Robinow (93) felt that, apart from a certain group, he was speaking for a consensus when he wrote, "The chromatin bodies of bacteria lie separately in the cytoplasm. They contain Feulgen positive material but are not markedly basophilic either between or during divisions. Chro-

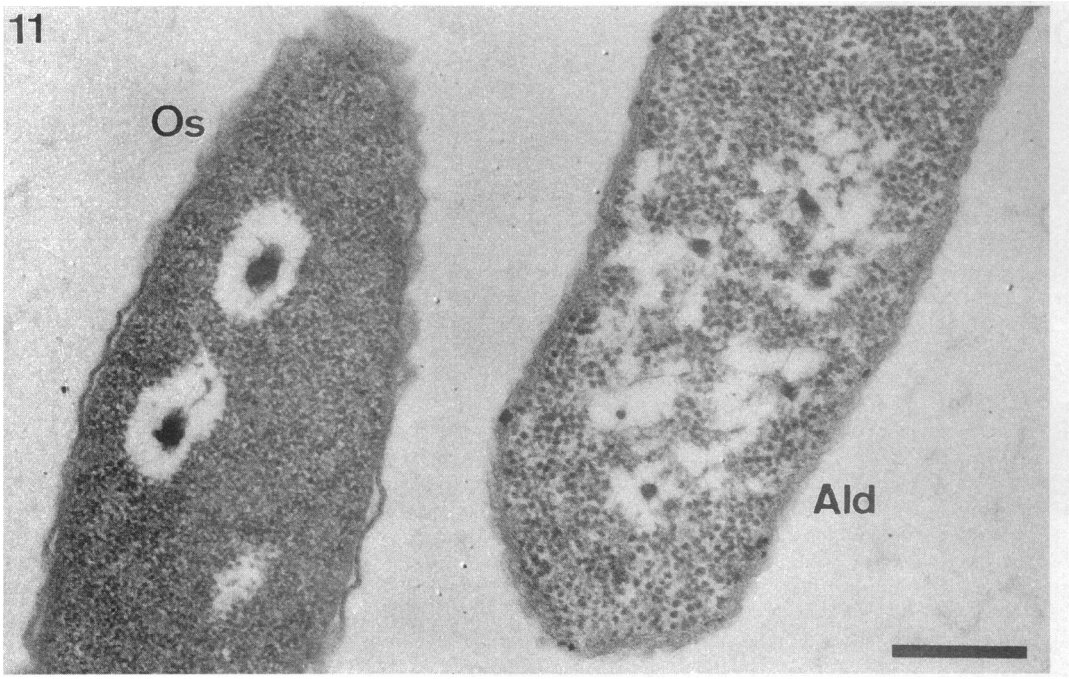


FIG. 11. Different aspects of the cytoplasm after aldehyde and conventional osmium fixation. In both cases the DNA-plasm is aggregated, because no uranyl acetate postfixation was used. A growing culture of *E. coli* was split into two aliquots; one was fixed in 1% osmium tetroxide, and the other was fixed in 1% glutaraldehyde. After fixation and washing, the aliquots were pooled and further processed. The thin sections were stained with uranyl acetate to produce sufficient contrast with the aldehyde-fixed cells. Note the distinctly visible ribosomes in the aldehyde-fixed cell. As already noted in the legend to Fig. 10, the osmium fixation is apparently accompanied by a strong staining of the ground plasma, which might be sufficiently intense to obscure the ribosomes. Ribosome-free spaces are thus hidden by the osmium fixation. These spaces contain DNA and form excrescences of the nucleoid as shown in Fig. 14. Bar, 0.3 μm. The micrograph was prepared in Basel in collaboration with Renate Gyalog.

matin bodies lack a demonstrable membrane, have the same texture and staining properties between and during divisions and divide directly in the sense that they are what they are at all times and do not, like chromosome-nuclei, pass through phases where they are something different. It is clear that chromatin bodies are not comparable to the chromosome nuclei of higher organisms and of protists. . . ." From this declaration and others in similar vein made over the next few years (95, 97), it may be concluded that we did not remotely expect the electron microscope to reveal bacterial nucleoids in the guise of tiny conventional nuclei complete with envelopes, sets of chromosomes, centrioles, and spindles. When, 6 years later, the work of Wollman and Jacob, Kellenberger's group, and Kleinschmidt and Lang had been done and the singular nature of the bacterial chromosome had just begun to be understood, a review discussing the new insights and hypotheses (by Robinow [97], assisted by John Marak) presented photomicrographs of HCl-Giemsa-stained nucleoids alongside RK→U-type electron micrographs from the London, Ontario, laboratory as two different aspects of the same kind of objects, which, lacking texture resolvable in the light microscope, had now been shown to be composed (as it appeared at the time) of fine twisted fibrils. Thus, there was then no feeling of a break with previous notions of the nature of bacterial nucleoids, only one of relief that their nature had been rendered more intelligible. That was long ago, but nothing that we have learned in the years since then obliges us to change that opinion. The nucleoids or "nuclear analogues" (39) detected by the light microscopist and the electron microscopist are still seen as the typical configuration that a DNA-plasm containing the bacterial genome assumes in growing and rapidly dividing cells. As before, under suitable conditions, phase-contrast microscopy immediately reveals the features in which they most obviously differ from the mitotic chromosomes of nuclei of eukaryotes, such as their cloudlike softness, fast-changing contours, and low optical density.

CHANGING VIEWS OF THE NUCLEOID

Shortcomings of RK Fixation, and How They Were Overcome by Autoradiography

In most osmium-fixed cells the ribosomes are not well resolved (Fig. 11), apparently because the cytoplasmic ground plasma (surrounding the ribosomes) is also very strongly stained. Exceptions, showing a less strong stain, have also been observed (Fig. 10B). However, the RK protocol for preparing bacteria for electron microscopy was successful insofar as it preserved their DNA-plasms in a finely fibrous state. Before long it became obvious, however, that the area occupied by a detectable DNA-plasm in sections of RK→U-fixed bacteria is smaller and more confined than phase-contrast microscopy of living bacteria would lead us to expect. A certain price, it seems, has to be paid for good preservation. But that was not all. Another, more puzzling aspect of RK→U-fixed nucleoids was the apparent lack of correspondence between their morphology and what had been learned about their mode of functioning in the years since the RK fixative had first been accepted as the standard in bacterial caryology. The salient point of that story, as far as it concerns our subject, is the

knowledge, derived from the biochemical analysis of growing bacteria, that because of the absence of a nuclear envelope, transcription is immediately followed by translation and that newly formed mRNA is at once occupied by ribosomes. Bringing biochemical data home to morphologists, Miller et al. (76), working with material isolated from growing *E. coli* cells, demonstrated in electron micrographs how ribosomes line up on mRNA to form polysomes. Strangely, however, these relationships are not reflected in the fine structure of the DNA-plasm of RK-fixed nucleoids. This situation was perceived as a challenge by Ryter (the very "R" of RK) and Chang (104). In the introduction to their stimulating paper, the authors remind us that DNA-ribosome complexes are absent from the DNA-plasms of RK-fixed bacteria and that "If these pictures are not the result of artefacts, they suggest strongly that genes transcribing messenger RNA are located at the periphery of the nucleus and not inside it. . . ." This interpretation agrees, in part at least, with the results obtained by Caro and Forro (14) in a pioneering study with high-resolution autoradiography at the electron-microscopic level, i.e., that ". . . a uridine H^3-label was first incorporated in the nuclear region or in a narrow band of cytoplasm surrounding it." Ryter and Chang likewise tested their assumption with the help of autoradiography and reported that the statistical analysis of their results indicated that in growing bacteria ". . . the sites of RNA synthesis are located at the periphery of nuclear areas and far out in the cytoplasm" (see Fig. 13A). Furthermore, and most importantly, the authors found evidence ". . . that a portion of the bacterial chromosome is indeed spread out into the cytoplasm and that the amount of this extranuclear DNA seems to be related to the growth rate." This is in accord with what straight electron microscopy had, in favorable cases, revealed earlier, namely that ". . . in many places fibrils from the nucleoplasm enter the cytoplasm" (120). However, even at the time of the announcement of the discovery by Ryter and Chang (104) of the function of such extensions, it was still possible that their usual absence from standard RK→U images of sections merely reflected that shortcoming to which we drew attention at the beginning of this section, namely that osmium fixation, even in the context of the RK→U protocol, changed the nucleoids so that they appeared confined to smaller areas (volumes) than they occupied during life, thus conceivably hiding the DNA-mRNA complexes generated at their periphery (see below). In the end, as we shall see, these reservations proved unjustified. Meanwhile, our own advance into the promised borderlands of RNA synthesis did not get under way until we decided to avoid chemical fixatives, with their potential of distorting the normal configuration of nucleoids and of the finer structures of the DNA-plasm, and changed from the RK→U procedure to cryofixation followed by freeze-substitution (CFS), a method that had already proved its worth in other spheres of study, e.g., fungal cytology (48, 74).

Let us stress here that our review has narrowly focused on the nucleoid. At the same time, the era of the electron microscopy of sections of chemically fixed bacteria has also seen much work on the bacterial cytoplasm, endospores, cell wall, plasma membrane, flagella, and bacteriophages. Much of the original documentation of such work has been collected and critically considered by van Iterson in two volumes (121, 122).

New View of the Nucleoid Provided by CFS Treatment

The CFS regime that we have adopted (47) avoids chemical prefixation and dispenses with cryoprotectants commonly used in freeze-fracturing procedures. Particular care is taken to avoid oxygen starvation. To this end the cells in aerated cultures in liquid media are concentrated by filtration without interruption of the active air supply. The thin layer of cells deposited on the filter is rapidly transferred to thin cigarette paper and frozen in a way which embeds bacteria within milliseconds in a layer of vitrified ice not exceeding 10 to 20 nm thick. It seems that by this procedure bacteria are frozen so rapidly that their contents are immobilized before any putative leakage of ions to the outside, a common event in chemical fixation, can occur.

Electron microscopy of sections of *E. coli* and *B. subtilis* preserved by CFS have revealed novel features of the texture and geometry of DNA-plasms, which are described by Hobot et al. (46, 47), Bohrmann et al. (6, 8), Dürrenberger et al. (25), and Kellenberger (60). Despite the avoidance of chemical stabilizers, there is no trace of the aggregation artifact which provided the stimulus for the design of RK→U fixation 35 years ago. On the contrary, the DNA-plasm of nucleoids in sections of bacteria preserved by the CFS procedure appears as a system of inconspicuous areas of ribosome-free spaces occupied by a fine, seemingly random granulation (Fig. 12A). Gone and still unaccounted for are the distinct fibrils arranged in loose (because during life highly hydrated) twisted bundles and arcs that were the hallmark of uranyl acetate postfixation (Fig. 10B and 12). The vagueness of the texture of the DNA-plasms in sections of CFS preparations at first made it difficult to trace the boundaries of territories occupied by nucleoids, let alone their three-dimensional configuration. Techniques of immunolabeling and immunostaining, described by Hobot et al. (46) and Dürrenberger et al. (25), and the reconstruction of nucleoids from a series of sections of labeled bacteria (8) have revealed details of the shape of nucleoids that are beyond the resolving power of the light microscope. A nucleoid of *E. coli* or *B. subtilis*, to go no further at this point, now appears to us to comprise two different regions (Fig. 13B and 14). It has a confined interior, which is drawn out at its periphery into a halo of numerous long, thin, probably branched coralline projections that, as correctly surmised by Ryter and Chang (104), extend deep into the cytoplasm. What we see in living, growing bacteria under the microscope, e.g., under phase-contrast conditions and/or DAPI fluorescence staining, is only the central bulk of a nucleoid whose delicate peripheral extensions remain unresolved. We have successfully simulated the postulated limitations of the light microscope by using a pinhole camera to photograph a cryofixed bacterium reconstructed from stacks of tracings of sections of it on thin, slightly colored foils with the ribosome-free areas cut out. In the image obtained in this way, the thin projections from the surface of the DNA-plasm were no longer distinguishable, thus reducing the configuration of the nucleoid to the kind of shape familiar from phase-contrast photomicrographs (8). The same reasoning, mutatis mutandis, obviously holds true for photomicrographs of nucleoids in fixed and stained preparations. We did not come across unexpected structures in the cytoplasm of CFS-treated bacteria and noted the absence from it of the formerly frequently reported "mesosomes." This finding is in agreement with observations on freeze-fractured cryofixed and cryosectioned bacteria by Higgins et al. (41) and Dubochet et al. (24). Aldehyde- or CSF-fixed cells show the excrescences as clearings or ribosome-free spaces (see Fig. 14). The presence therein of DNA has been revealed only by the new method of immunostaining (8).

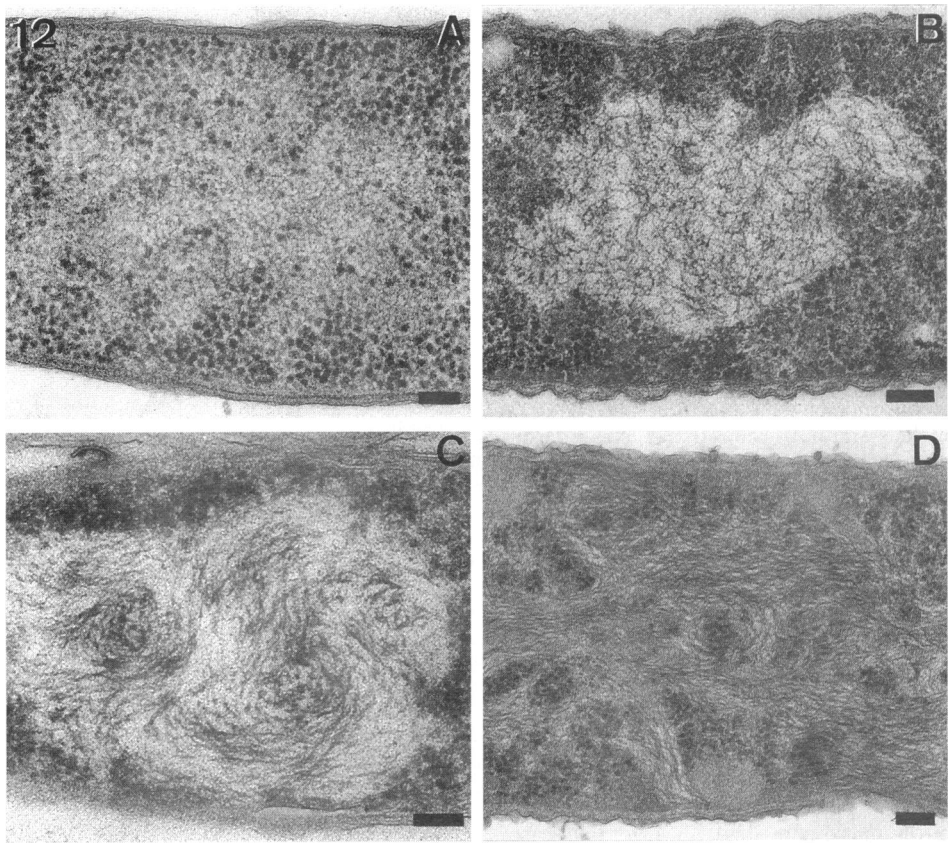


FIG. 12. According to the fixation procedure used, DNA-plasms show very different fine structures. (A and B) Nucleoids of *E. coli*. (C and D) Parts of an artificially enlarged vegetative bacteriophage T4 DNA pool that occurs, e.g., in mutants blocked at the prehead stage (as here) or by inhibition of the expression of late proteins by chloramphenicol, puromycin, or other procedures involving amino acid-requiring mutants. (A) Nucleoids after CFS treatment contain a DNA-plasm of the fine granular type, which is interpreted as reflecting supercoiled chromatin. (B) The network type of DNA-plasm obtained after osmium fixation under strict RK conditions and postfixation in uranyl acetate. Compare also with Fig. 7 of reference 105. (C and D) Whorl-type DNA-plasm after cytoplasmic fixation with glutaraldehyde followed by a DNA fixation in uranyl acetate. Bar, 0.1 μm. Micrographs were prepared in Basel in collaboration with Renate Gyalog.

FUNCTION-STRUCTURE RELATIONSHIPS

Tracing Sites of DNA and mRNA in Sections of CFS-Treated Bacteria

DNA. In sections of CFS-preserved bacteria, nucleoids are seen as rather vaguely defined areas free of ribosomes. The images of (anti-DNA) immunostained nucleoids (Fig. 14C) reflect what we had previously learned from three-dimensional reconstructions of sectioned (not immunostained) CFS-preserved bacteria, namely that slender projections of DNA-plasm extend from the surface of nucleoids into the cytoplasm.

RNA. We have used conventional procedures of immunolabeling in looking for sites of RNA polymerase, topoisomerase I, and the "histonelike" protein HU (25) and, in agreement with Ryter and Chang (104), have found labels for all three compounds in areas of cytoplasm adjacent to nucleoids. The ca. 20% of gold label on small islands of apparently pure cytoplasm not adjacent to nucleoids is now regarded as representing cross-sections of nuclear projections or excrescences. This small proportion of label has been interpreted in the literature as suggesting a binding of HU to ribosomes (108).

The Shape of Nucleoids Reflects the Level of Their Metabolic Activities

The delicate excrescences, which we described above as arising from the surface of nucleoids, are rarely visible after osmium fixation, but their presence among the ribosomes of the cytoplasm, inferred from the distribution of silver grains in autoradiographs, can be more precisely pinpointed by immunolabeling of sections of CFS-fixed bacteria; finally, in such sections, profiles of the delicate projections are made directly visible by immunostaining. The excrescences obviously represent stretches of the genome that are engaged in transcription. We imagine them to be single or multiple loops which, having done their work, may retreat into central bulk of the nucleoid. As Ryter and Chang (104), who had come to similar conclusions, have plausibly suggested, "This (continuous) movement is perhaps partially responsible for the irregular shape of the nuclear area in growing bacteria." This view of nucleoid morphology would also account for the confinement of nucleoids in the nearly spherical shapes that, as we have shown repeatedly (see reference 59 and references therein), they acquire under the influence of any agent or procedure that inhibits protein synthesis, thereby doing away with the need for the transcription-translation machinery.

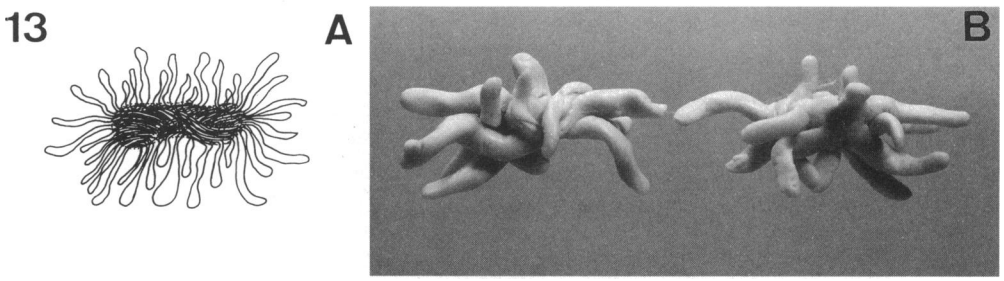


FIG. 13. Models of bacterial nucleoids in metabolically active states. (A) Model A of Ryter and Chang (104) is deduced indirectly from the autoradiographically determined sites of mRNA synthesis. (B) Model B of Kellenberger (60) is based on the observation of ribosome-free spaces in the cytoplasm (see Fig. 14A and B), which were found to contain DNA by the new immunostain procedure (8) as shown in Fig. 14C. The sites where the RNA polymerase is located, as detected by immunogold labeling, are also outside the bulk part of the nucleoid (25). As explained in the legend to Fig. 11, the ribosome-free spaces in the cytoplasm can usually not be detected in osmium-fixed cells (as were used in the experiments for establishing model A). They were, however, easily visible in the CFS-fixed cells used as basis for model B. The two models are basically identical in illustrating that transcription of the bacterial chromosome occurs not inside the bulk nucloid but on loops or excrescences that extend far into the cytoplasm. Model A reprinted from reference 104 with permission.

To return to normal conditions of growth, the demonstration of projections from the surface of nucleoids that extend far into the cytoplasm strengthens the plausibility of speculations that contacts, perhaps at several points, between nucleoids and the plasma membrane itself or structures situated on it (49) might be mediated through the excrescences. Such contacts are proposed to play an important part in DNA partition (50). It should also prove rewarding to use CFS fixation in fresh studies of the effects of different rates of growth on the configurations of nucleoids, while their number per cell is most easily obtained after DAPI fluorescence staining of fixed bacteria (45).

Ill-Defined Interlinked "Nucleoids"

As clearly and repeatedly demonstrated by Delaporte (22), the nucleoid ("noyau") of certain large bacteria regularly encountered in the gut of tadpoles takes the form of a long, wavy "filament axial" far larger than a bacterial genome of the usual length in an in vivo conformation. At present such nucleoids are best regarded as closely packed multiple (transcribing) copies of the basic genome of the large bacteria in question. In ordinary bacteria, too, a variety of damaging influences may lead to the formation of long axial cords or more dispersed arrangements of DNA-plasms. Such configurations are encountered during the initial stages of infection with bacteriophages and during recovery from exposure to low doses of UV or ionizing radiation (55, 103). Other examples are provided by bacteria transformed into "large bodies," which attracted attention during the early days of the widespread use of penicillin. We suggest that in instances of this kind the designation of "the nucleoid" be avoided and that "sites occupied by dispersed DNA-plasm" be used instead.

Looking for a Resting DNA-Plasm

In normally growing cultures, DNA replication proceeds simultaneously along three replication forks and transcription-translation does not even pause during cell division. Despite this, we habitually speak of "the genome," "the chromosome," or "the nucleoid," although in the ordinary course of our work we never encounter a truly single, truly resting example of these entities. This limitation draws attention to another potential field of study in which the CFS procedure is likely to reward the first investigator to use it to explore a true terra incognita, namely, the physical state of the DNA of spores, whose fine structure in even the best-preserved samples still does not reveal levels of morphological complexity exceeding that of a pane of ground glass. Lacking detectable metabolism but poised to start metabolism within minutes of being placed in a suitable environment, spores, according to Seward (112), may lie dormant for up to 2,000 years and probably much longer. This state of bacterial life may provide our chance of encountering a normal, truly resting genome (or several of them, as suggested by Johnston and Young [52] and Hauser and Karamata [38]), which, unlike genomes in stationary-phase cells from crowded cultures, has not been reduced to a state of rest merely by exhaustion of nutrients in the culture medium. Equally interesting would be CFS studies of developing spores preserved at the time when their DNA is undergoing the physical and chemical changes involved in rendering it fit to survive long periods of dormancy. Inviting, too, are studies of presumed changes in fine structure accompanying the resumption of its normal activities by the DNA of germinating spores.

GIANT BACTERIA

Strange motile organisms of uncertain nature found in the gut of tropical herbivorous surgeon fish (*Acanthuridae*: *Teleostei*) by Fishelson et al. (27) and placed by them in the new genus *Epulopiscis* have also been encountered in surgeon fish from the region of the Great Barrier Reef. Electron microscopy of sections of the largest of these kinds of symbionts by Clements and Bullivant (17) has demonstrated that "epulos," as they are colloquially known, have typical prokaryotic aggregation-sensitive DNA-plasm and possess bacterium-type flagella and that they are, in fact, giant bacteria measuring up to 500 μm in length.

K. D. Clements, James Cook University, Townsville, Australia, has kindly provided us, via Esther Angert (2), Department of Biology, University of Indiana, Bloomington, with a sample of several kinds of these bacterial symbionts preserved in 80% ethanol. We have stained the chromatin of these epulos with HCl-Giemsa (Fig. 15) and by the Feulgen procedure, which have given concordant results, as have experiments with further samples of epulos preserved with Schaudinn's mercuric chloride-alcohol-acetic acid fixative. Our results indicate that DNA-plasms of mature epulos are disposed in a loose net-like pattern close to the cell wall. Epulos do not multiply by transverse fission but, as a rule, by the generation of two (or

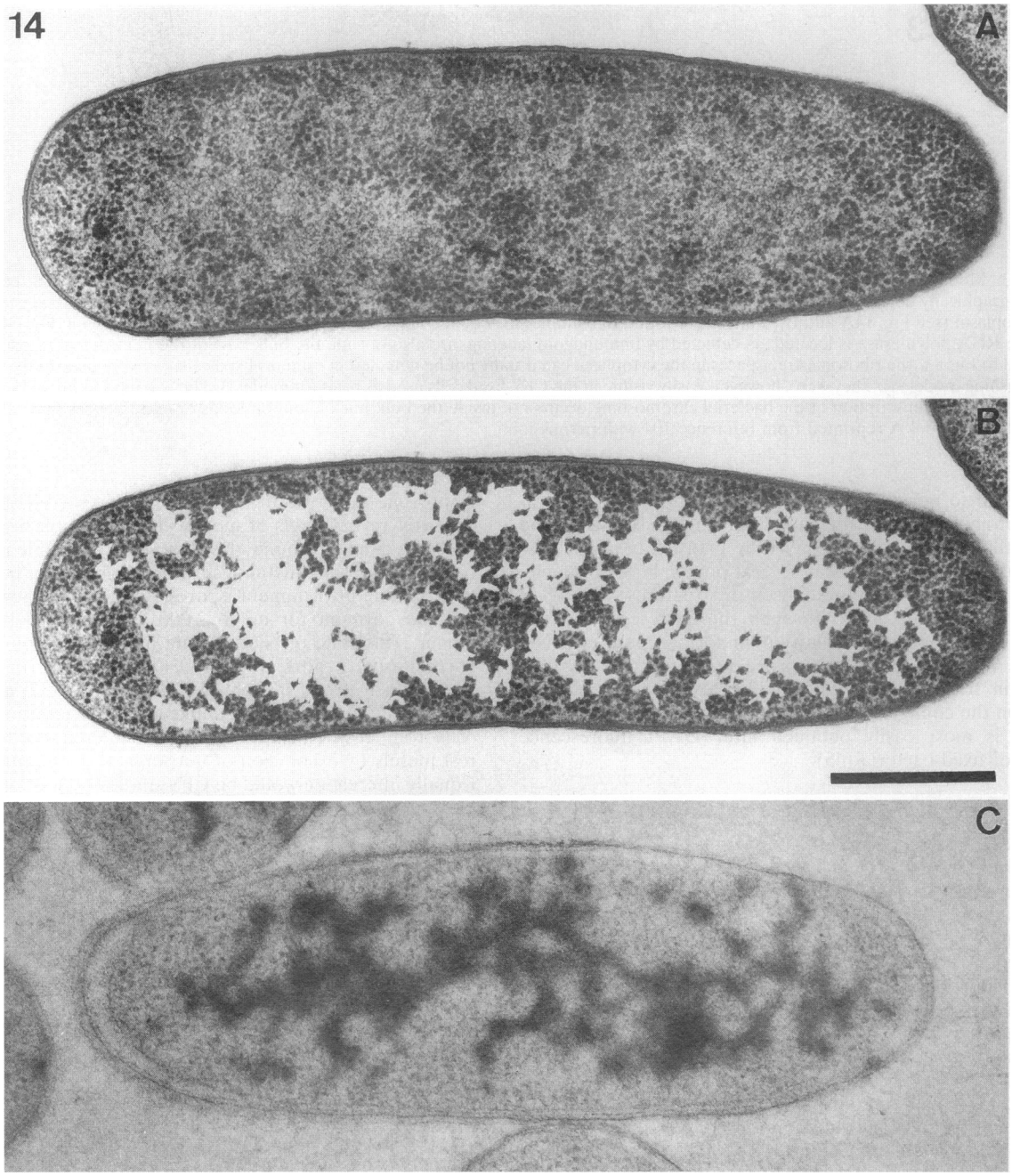


FIG. 14. The nucleoid with its excrecences in thin sections of growing *E. coli* after CFS. Panels A and B show the same section; in panel B the ribosome-free spaces were enhanced by coloring by hand. Panel C contains a similar cell, immunostained specifically for DNA by the procedure of Bohrmann et al. (8). The micrograph in panel A was prepared in Basel with Markus Dürrenberger and Werner Villiger, and the micrograph in panel C was also prepared in Basel by Bernd Bohrmann.

more) daughter organisms, end to end or side by side, within the body of a mother epulo (2, 17), from which, at maturity, they are released by disintegration of the mother organism. Much remains to be learned about these spectacular bacteria, which have so far defied attempts to culture them in vitro. Having devoted most of our space to *E. coli* and *Bacillus* cells, we thought it fitting, as far as bacteria go, to end this review with "a view of things to come."

A SIDEWAYS GLANCE AT THE CHROMOSOMES OF DINOFLAGELLATES

Over the past 30 years the chromosomes of one group of protozoa, the dinoflagellates, have received a great deal of attention because in electron micrographs of sections, and without having undergone any form of artificial unraveling, these chromosomes display at all phases of the nuclear cycle a uniquely ordered fibrillar organization that has not so far been

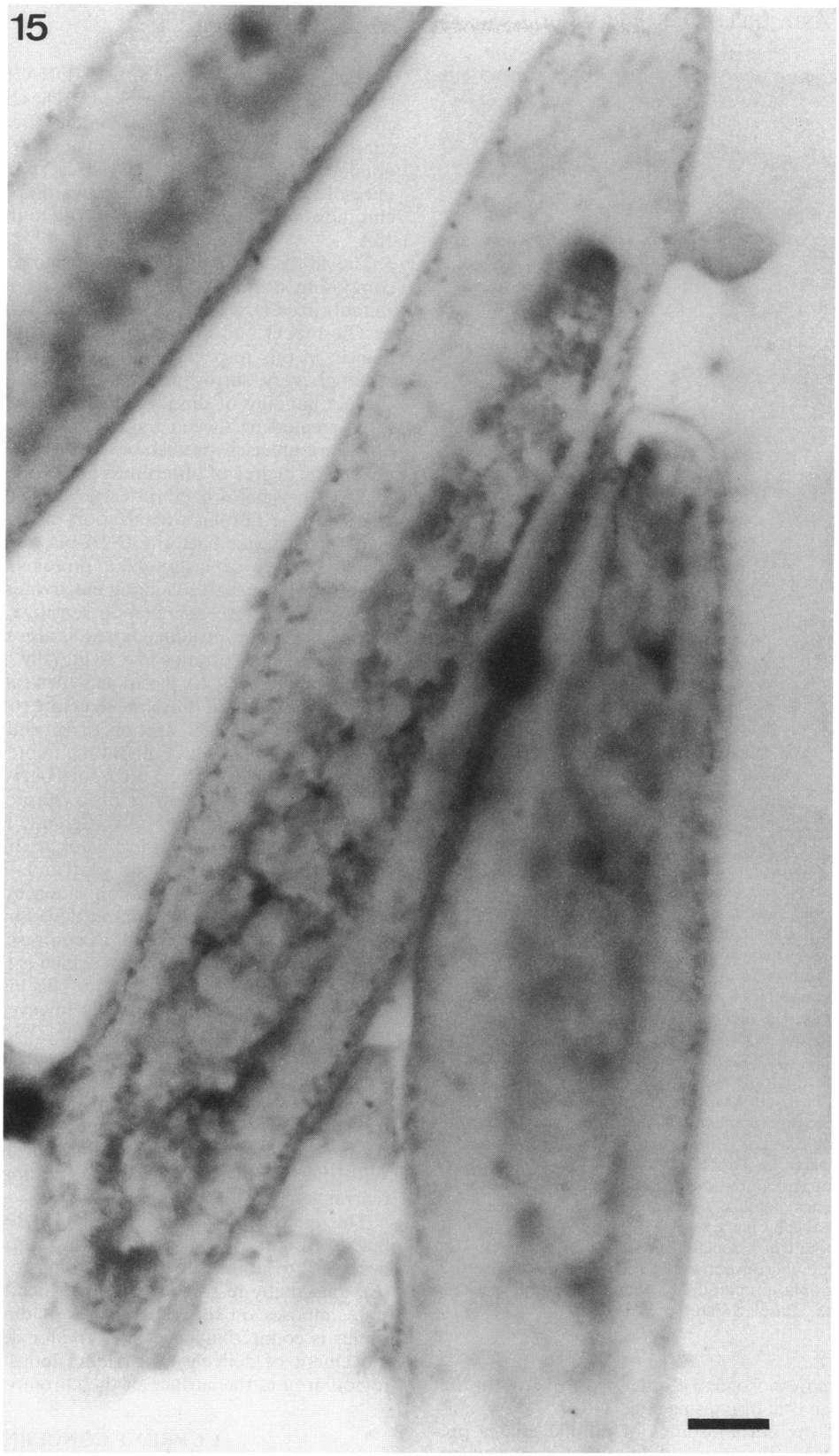


FIG. 15. Light micrograph of the giant bacterium *E. fishelsoni* in a sample of symbionts of the surgeon fish kindly provided by K. D. Clements and Esther Angert. The bacterium was stained with HCl-Giemsa. All epulos in this figure harbor daughter organisms. The cell wall is clearly visible as a soft gray contour along the left edge and at the top right of the central bacterium. A net-like pattern of pieces of chromatin lines what presumably is the inner surface of the plasma membrane close to the cell wall. The much more strongly stained patches of chromatin of the daughter organism appear to be arranged on a cylindrical surface, which we believe to be that of a plasma membrane beneath the inner face of its own cell wall. A short stretch of such a wall of a daughter bacterium is faintly visible in the neighboring epulo to the right and above the dark material in the center of the photograph. Bar, 5 μm (i.e., the length of an average *E. coli* cell at this magnification).

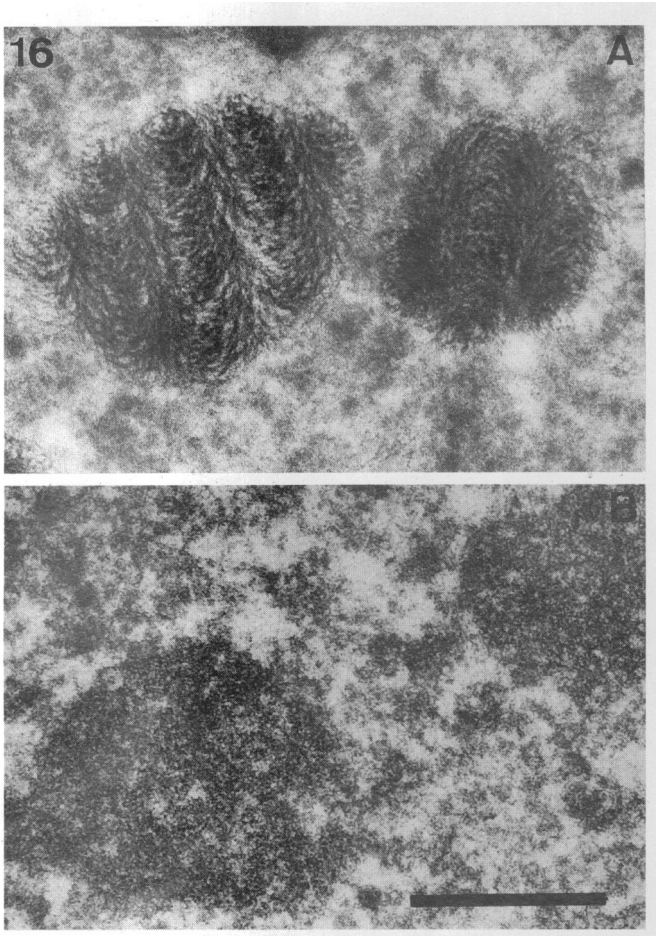


FIG. 16. Permanently condensed "chromosomes" of dinoflagellates and of a euglenoid flagellate. In the permanently condensed chromosomes of the dinoflagellates, the DNA-plasm is always in the form of a liquid crystal (A), even when prepared by the CFS technique, whereas this fine structure is never found in euglenas (B) or in other histone-containing chromatins of higher cells, totally independent of the method of fixation used. Kellenberger and Arnold-Schulz-Gahmen (61) explain this as follows. DNA-plasms of the whorl type (liquid crystalline [Fig. 10B, 12C and D; see also panel A]) are produced only with LP-chromatins of which the supercoil is relaxed, either naturally (as in the metabolically inactive chromosomes of dinoflagellates) or artifactually (by the action of chemical fixatives, which react with proteins but are not able to cross-link the LP-chromatin). This latter situation is found in bacteria. This LP-chromatin is characterized by low relative contents of protein partners. Large amounts of histones in the form of nucleosomes stabilize the supercoiled conditions, which persist even in metabolically inert regions of the HP-chromatin. By virtue of its large amount of associated proteins, HP-chromatin can also be cross-linked by all conventionally used cytological fixatives. Bar, 0.5 μm. Micrographs prepared and kindly supplied by Bernd Bohrmann, Biozentrum, Basel, Switzerland.

encountered in the chromosomes of other protozoa or, for that matter, higher eukaryotic organisms (Fig. 16).

The structure of the chromosomes of dinoflagellates presents a puzzle which has recently found a satisfactory explanation in the notion that these chromosomes ought to be regarded as liquid crystals (see references 10, 32, 70, 88, and 115 and references therein). In successive layers, piled on top of each other at right angles to the long axes of these chromosomes, the orientation of the DNA fibrils, which run parallel to each other, changes direction in an orderly manner. The characteristic appearance of DNA fibrils seemingly arranged in steep, closely packed arches is now seen as the consequence of oblique sectioning. Clear evidence of the continuity of the DNA fiber has, however, not yet been obtained. It is assumed that it is maintained by (as yet unresolved) loops extending outward from the bulk of the chromosome in a fashion comparable to that illustrated in Fig. 13A.

The fibrils in dinoflagellate chromosomes resemble those composing steeply curved arches, swirls, and twisted cords of suitably fixed DNA-plasms of bacteria, as has been pointed out by Giesbrecht and Drews (33–35), but the degree of resemblance, in this respect, between dinoflagellates and bacteria, although very surprising, must not be overestimated. The distinct banding of dinoflagellate chromosomes and the patterns created by the enigmatic ordering of their constituent fibrils are precisely predictable features, whereas, as discussed above, the degree of orderliness in the published fiber patterns of DNA-plasms of bacteria varies between laboratories. The characteristic fibrillar organization of dinoflagellate chromosomes, which, like bacterial DNA-plasms, have only relatively small amounts of histone-like proteins (40, 89, 125), is a hitherto unique feature among eukaryotes. The fact that their DNA-plasm is also aggregation sensitive (19), like the other chromatins of low protein content, is also noteworthy (3). That a dinoflagellate chromosome is literally "a different animal" from a bacterial DNA-plasm was illustrated by the preservation of the natural fibrillar structure of the dinoflagellate chromosome by CFS fixation, a procedure which deprives bacterial DNA-plasms of all but the faintest traces of a fibrous organization. This striking difference between the responses of two different representatives of low-protein chromatin to the nonchemical CFS procedure is currently explained as follows. The chromosomes of dinoflagellates are already natively existing "natural" liquid crystals (70) and remain in this state during the course of adequate fixation by chemical means as well as during the vitrification achieved by CFS fixation (3, 6, 32). DNA-plasms of bacteria, in contrast, are affected by the loss of ions from the cytoplasm, which is the immediate result of fixation with osmium tetroxide. This loss, together with the inactivation of enzymes crucially involved in producing and maintaining supercoiling, allows the DNA to relax its supercoiled state, which, according to the well-known data obtained by Pettijohn and coworkers (12, 84, 85), is its normal condition in growing bacteria. At this point the uncoiled DNA fibrils can recast themselves into the state of an artifactual fibrous liquid crystal before they are fixed and stabilized by uranyl acetate. With the CFS fixation technique, an opportunity for such a rearrangement does not arise.

The nuclear physiology of dinoflagellates is not our concern, but to round off this detour into protozoology it seems necessary to point out that according to recent observations (6), the greatly restricted mobility which the liquid-crystalline state imposes on the DNA fibrils of dinoflagellate chromosomes is counterbalanced by a greater degree of freedom of movement of actively transcribing loops of DNA in a halo region around the surface of the chromosomes (113, 114).

CURRENT CONCERNS

A long path has been followed since the time when, in the late 1930s, the existence of DNA in bacterial cells was confirmed by cytochemical light microscopy, a finding that immediately suggested the existence of bacterial genetics. It became increasingly clear that the bacterial nucleoid is not a static, morphologically precisely definable entity or compartment but

something very dynamic, which, during growing and metabolizing phases of the cell, undergoes variations of shape, chiefly in response to continuous activities of transcription. Therefore, interest in descriptive morphology, still important in the studies of the nuclei of eukaryotes, waned and was superseded by intense interest in functional mechanisms with their fine-structure implications at the molecular level.

A recent Keystone symposium on the bacterial chromosome (102) was unanimously held to have been highly successful owing to the wide range of subjects offered for discussion and the clear presentations of advances made during the last few years. Those attending returned from the conference with heightened awareness of the state of the art used in current studies of the chromosomal functions and activities.

This is not the place for summarizing this symposium; we will try, however, to emphasize briefly the links with some of the matters that we have discussed above. We will supply only a few references, mostly to reviews that deal with replication, transcription, and separation and partition of the genetic material into daughter cells or into sites which would be daughter cells if septations had not been inhibited or altered. Despite their high interest, we must omit all the purely genetic mechanisms, such as initiation of replication, mutagenesis, recombination, genetic transfers, and transpositions, because of their lesser relevance to the problems treated in the present review.

DNA-binding proteins, often histone-like, such as HU, H-NS, and IHF, in association with the various topoisomerases have been found to play important roles in modulating chromosomal functions (31, 80). The major involvement of these proteins departs increasingly from that of the eukaryotic histones, with their rather static function of compaction. At the same time, however, the mechanism of supercoil compaction of the chromatins with low protein contents (61) becomes an urgent question. What is the fine structure and the in vivo behavior of the postulated bacterial compactosomes which are found to be so very fragile when compared with the stable eukaryotic nucleosomes with their solid, histone core? Interest in bacteria has increased because LP (low-protein) chromatin has also been found in viruses, mitochondria, plastids, and the chromosomes of dinoflagellates (3).

The separation and partition of chromosomes into daughter cells has received much attention and has been studied intensively by combining morphological observations of DAPI-stained material with the use of genetic tools. Mutants which affect these activities have been collected and investigated (43, 71, 118).

Cellular septation is governed by the fibrous protein FtsZ of *E. coli* (72, 78). In vivo this protein was located at the future sites of septation, and in vitro it could be polymerized into beautiful rings. Correlations of partition with cell septation are to be expected but had not been detected. Surprising but not yet confirmed are recent observations (82) on a mutant of *E. coli* which overexpresses the *mre* genes and reveals dividing cells that are connected by a thick bundle of fibers. Cytoplasmic fibers, tentatively regarded as microtubuli, have previously been encountered in *Proteus mirabilis* (123); the possibility of these tubuli being originated through a (defective?) prophage has independently been confirmed (117a).

To push putative analogies with the chromosome partition in eukaryotes even further, an *E. coli* protein, MukB, was discovered and investigated (43, 44); this protein has structural and functional analogies with the protein family of myosin, dynein, and kinesin, which are all involved in intracellular movements, such as muscle contraction and flagellar movement.

The unique feature of bacteria (and their phages) of permitting in vivo and in vitro experiments with about equal precision and relevance gives us and many participants of the symposium great hopes that these experimental systems will continue to provide fundamental insights into the basic phenomena of life.

ACKNOWLEDGMENTS

Carl Robinow is indebted for counsel and support to R. G. E. Murray, a fellow bacterial anatomist, his colleague and friend for more than forty years. Both (retired) authors thank Dimitri Karamata for hospitality in his Institute (IGBM) during the later years of gestation of this review. We greatly appreciate the photographic work by Marlies Zoller and her crew at the Biozentrum in Basel. We are grateful for instruction and help in the use of the EndNote program by Marlies Maeder and Regula Niederhauser in Basel and Nadine Thomas in Lausanne and for some help with the presentation of the text.

REFERENCES

1. **Anderson, E. S., J. A. Armstrong, and J. S. F. Niven.** 1959. Fluorescence microscopy: observation of virus growth with aminoacridines. Symp. Soc. Gen. Microbiol. **9**:224–255.
2. **Angert, E. R., K. D. Clements, and N. R. Pace.** 1993. The largest bacterium. Nature (London) **362**:239–241.
3. **Arnold-Schulz-Gahmen, B., B. Bohrmann, L. Falquet, R. Gyalog, R. Johansen, M. Maeder, J. Pelzer, and E. Kellenberger.** Demonstration of the existence and broad occurrence of chromatins with low protein contents. Submitted for publication.
4. **Badian, J.** 1933. Eine zytologische Untersuchung über das Chromatin und den Entwicklungszyklus der Bakterien. Arch. Mikrobiol. **4**:409–418.
5. **Baker, J.** 1942. Some aspects of cytological technique, p. 1–27. *In* G. Bourne (ed.), Cytology and cell physiology. Clarendon Press, Oxford.
6. **Bohrmann, B.** 1992. Studies on the cellular localization, organization and packing density of transcribing and resting chromatin. Ph.D. thesis, University of Basel, Basel, Switzerland.
7. **Bohrmann, B., M. Haider, and E. Kellenberger.** 1993. Concentration evaluation of chromatin in unstained resin-embedded sections by means of low dose ratio contrast imaging in STEM. Ultramicroscopy **49**:235–251.
8. **Bohrmann, B., W. Villiger, R. Johansen, and E. Kellenberger.** 1991. Coralline shape of the bacterial nucleoid after cryofixation. J. Bacteriol. **173**:3149–3158.
9. **Borysko, E.** 1956. Recent developments in methacrylate embedding. I. A study of the polymerization damage phenomenon by phase contrast microscopy. J. Biophys. Biochem. Cytol. **2**:3–14.
10. **Bouligand, Y., M.-O. Soyer, and S. Puiseux-Dao.** 1968. La structure fibrillaire et l'orientation des chromosomes chez les dinoflagellés. Chromosoma **24**:251–287.
11. **Brock, T. D.** 1988. The bacterial nucleus: a history. Microbiol. Rev. **52**:397–411.
12. **Broyles, S. S., and D. E. Pettijohn.** 1986. Interaction of the *Escherichia coli* HU protein with DNA. Evidence for formation of nucleosome-like structures with altered DNA helical pitch. J. Mol. Biol. **187**:47–60.
13. **Cairns, J.** 1963. The bacterial chromosome and its manner of replication as seen by autoradiography. J. Mol. Biol. **6**:208–213.
14. **Caro, L. G., and F. Forro.** 1961. Localization of macromolecules in *Escherichia coli*. II. RNA and its site of synthesis. J. Biophys. Biochem. Cytol. **9**:555–565.
15. **Cassel, W. A., and W. G. Hutchinson.** 1954. Nuclear studies on the smaller Myxophycea. Exp. Cell Res. **6**:134–150.
16. **Chapman, G. B., and J. Hillier.** 1953. Electron microscopy of ultrathin sections of bacteria. I. Cellular division in *Bacillus cereus*. J. Bacteriol. **66**:362–373.
17. **Clements, K. D., and S. Bullivant.** 1991. An unusual symbiont from the gut of surgeon fishes may be the largest known procaryote. J. Bacteriol. **173**:5359–5362.
18. **DeBary, A.** 1884. Vergleichende Morphologie und Biologie der Pilze, Mycetozoen und Bakterien. Wm. Engelmann, Leipzig, Germany.
19. **de Haller, G., E. Kellenberger, and C. Rouiller.** 1964. Etude au

microscope électronique des plasmas contenant de l'acide désoxyribonucléique. III. Variations ultra-structurales des chromosomes d'Amphidinium. J. Microsc. **3**:627–642.
20. **DeLamater, E.** 1954. Cytology of bacteria. II. The bacterial nucleus. Annu. Rev. Microbiol. **8**:23–46.
21. **Delaporte, B.** 1939–1940. Recherches cytologiques sur les bactéries et les cyanophycées. Rev. Gén. Bot. **51–52**:615–643, 689–708, 748–768, 112–160.
22. **Delaporte, B.** 1964. Etude descriptive de bactéries de très grandes dimensions. Ann. Inst. Pasteur (Paris) **107**:845–862.
23. **Delaporte, B.** 1969. Description de *Bacillus medusa*. C. R. Acad. Sci. Paris **269**:1129–1131.
24. **Dubochet, J., A. W. McDowall, B. Menge, E. N. Schmid, and K. G. Lickfeld.** 1983. Electron microscopy of frozen-hydrated bacteria. J. Bacteriol. **155**:381–390.
25. **Dürrenberger, M., M.-A. Bjornsti, T. Uetz, J. A. Hobot, and E. Kellenberger.** 1988. Intracellular localization of the histone-like protein HU in *Escherichia coli*. J. Bacteriol. **170**:4757–4768.
26. **Eiserling, F. A., and W. R. Romig.** 1962. Studies of *B. subtilis* bacteriophages. Structural characterization by electron microscopy. J. Ultrastruct. Res. **6**:540–546.
27. **Fishelson, L., W. L. Montgomery, and A. A. Myrberg.** 1985. A unique symbiosiy in the gut of tropical herbivorous surgeon fish (*Acanthuridae*: *teleostei*) from the Red Sea. Science **229**:49–51.
28. **Fritsch, F. E.** 1945. The structure and reproduction of the algae, vol. 2. Cambridge University Press, Cambridge.
29. **Fuhs, G. W.** 1965. Symposium on the fine structure and replication of bacteria and their parts. I. Fine structure and replication of bacterial nucleoids. Bacteriol. Rev. **29**:277–293.
30. **Fuhs, G. W.** 1969. The nuclear structures of protocaryotic organisms Bacteria and Cyanophyceae, p. 180. Protoplasmatologia (Handbuch der Protoplasmaforschung), vol. 5/4. Springer-Verlag KG, Berlin.
31. **Gama, M. J., A. Toussaint, and N. P. Higgins.** 1992. Stabilization of bacteriophage Mu repressor-operator complex by the Escherichia coli integration host factor protein. Mol. Microbiol. **6**:1715–1722.
32. **Gautier, A., L. Michel-Salamin, E. Tosi-Couture, A. W. McDowall, and J. Dubochet.** 1986. Electron microscopy of the chromosomes of dinoflagellates *in situ*: confirmation of Bouligand's liquid crystal hypothesis. J. Ultrastruct. Mol. Struct. Res. **97**:10–30.
33. **Giesbrecht, P.** 1962. Vergleichende Untersuchungen an den Chromosomen des Dinoflagellaten *Amphidinium elegans* und denen der Bakterien. Zentralbl. Bakteriol. I Orig. Abt. 1 B **187**:452–498.
34. **Giesbrecht, P.** 1965. Ueber die Tertiärstruktur der DNS in den Chromosomen lebender Zellen. Z. Naturforsch. **20b**:927–929.
35. **Giesbrecht, P., and G. Drews.** 1981. Die "Kernstrukturen" der Bakterien und ihre Beziehungen zu denen der "Mesokaryonten", p. 542–597. *In* H. Metzner (ed.), Die Zelle—Struktur and Funktion. Wissenschaft Verlagsges., Stuttgart, Germany.
36. **Glauert, H. M., G. E. Rogers, and R. H. Glauert.** 1956. A new embedding medium for electron microscopy. Nature (London) **178**:803.
37. **Hartman, P. E., and J. I. Payne.** 1954. Direct staining of the two types of nucleoproteins in *Escherichia coli*. J. Bacteriol. **68**:237–242.
38. **Hauser, P. M., and D. Karamata.** 1992. A method for the determination of bacterial spore DNA content based on isotopic labelling, spore germination and diphenylamine assay: ploidy of spores of several Bacillus species. Biochimie **74**:723–733.
39. **Hayes, W.** 1964. The genetics of bacteria and their viruses. Blackwell Scientific Publications, Oxford.
40. **Herzog, M., and M.-O. Soyer.** 1981. Distinctive features of dinoflagellate chromatin. Absence of nucleosomes in a primitive species of *Prorocentrum micans* E. Eur. J. Cell Biol. **23**:295–302.
41. **Higgins, M. L., H. C. Tsien, and L. Daneo-Moore.** 1976. Organization of mesosomes in fixed and unfixed cells. J. Bacteriol. **127**:1519–1523.
42. **Hillier, J., S. Mudd, and A. G. Smith.** 1949. Internal structure and nuclei in cells of *Escherichia coli* as shown by improved electron microscopy techniques. J. Bacteriol. **57**:319–335.
43. **Hiraga, S.** 1992. Chromosome and plasmid partitioning in *Escherichia coli*. Annu. Rev. Biochem. **61**:283–306.
44. **Hiraga, S.** 1993. Chromosome partition in *Escherichia coli*. Curr. Opin. Genet. Dev. **5**:789–801.
45. **Hiraga, S., H. Niki, T. Ogura, C. Ichinose, H. Mori, B. Ezaki, and A. Jaffé.** 1989. Chromosome partitioning in *Escherichia coli*: novel mutants producing anucleate cells. J. Bacteriol. **171**:1496–1505.
46. **Hobot, J. A., M.-A. Bjornsti, and E. Kellenberger.** 1987. Use of on-section immunolabeling and cryosubstitution for studies of bacterial DNA distribution. J. Bacteriol. **169**:2055–2062.
47. **Hobot, J. A., W. Villiger, J. Escaig, M. Maeder, A. Ryter, and E. Kellenberger.** 1985. Shape and fine structure of nucleoids observed on sections of ultrarapidly frozen und cryosubstituted bacteria. J. Bacteriol. **162**:960–971.
48. **Howard, R. J., and J. R. Aist.** 1979. Hyphal tip cell ultrastructure of the fungus *Fusarium*. Improved preservation by freeze-substitution. J. Ultrastruct. Res. **66**:224–234.
49. **Imamura, R., H. Niki, M. Kitaoka, K. Yamanaka, T. Ogura, and S. Hiraga.** 1992. Characterization of high molecular weights of complexes and polymers of cytoplasmic proteins in *Escherichia coli*. Res. Microbiol. **143**:743–753.
50. **Jacob, F., S. Brenner, and F. Cuzin.** 1963. On the regulation of DNA replication in bacteria. Cold Spring Harbor Symp. Quant. Biol. **28**:329–348.
51. **Jacobson, W., and M. Webb.** 1952. The two types of nucleoproteins during mitosis. Exp. Cell Res. **3**:163–183.
52. **Johnston, G. C., and I. E. Young.** 1972. Variability of DNA content in individual cells of Bacillus. Nature (London) New Biol. **238**:164–166.
53. **Kellenberger, E.** 1952. Les nucleoides d'*E. coli* étudiés à l'aide du microscope électronique. Experientia **8**:99–101.
54. **Kellenberger, E.** 1953. Les formes caractéristiques des nucléoides de *Escherichia coli* et leurs transformations dues à l'action d'agents mutagènes-inducteurs et de bactériophages, p. 45–66. *In* S. Mudd and G. Penso (ed.), Symposium Citologia Batterica (Roma), Supplemento Rendiconti Istituto Superiore di Sanita. Also obtainable (84-03,992) from University Microfilms International, Ann Arbor, Mich.
55. **Kellenberger, E.** 1955. Les transformations des nucléoides de *Escherichia coli* déclanchées par les rayons X. Experientia **11**: 305–307.
56. **Kellenberger, E.** 1960. The physical state of the bacterial nucleus. Symp. Soc. Gen. Microbiol. **10**:39–66.
57. **Kellenberger, E.** 1962. The study of natural and artificial DNA-plasms by thin sections, p. 233–250. *In* R. J. C. Harris (ed.), The interpretation of ultrastructure, vol. 1. Academic Press, Inc., New York.
58. **Kellenberger, E.** 1988. About the organization of condensed and decondensed non-eukaryotic DNA and the concept of vegetative DNA (a critical review). Biophys. Chem. **29**:51–62.
59. **Kellenberger, E.** 1989. Bacterial chromatin (a critical review of structure-function relationships), p. 3–25. *In* K. W. Adolph (ed.), Chromosomes: eukaryotic, prokaryotic and viral, vol. 3. CRC Press, Inc., Boca Raton, Fla.
60. **Kellenberger, E.** 1991. Functional consequences of improved structural information on bacterial nucleoids. Res. Microbiol. **142**:229–238.
61. **Kellenberger, E., and B. Arnold-Schulz-Gahmen.** 1992. Chromatins of low protein content: special features of their compaction and condensation. FEMS Microbiol. Lett. **100**:361–370.
62. **Kellenberger, E., and D. Bitterli.** 1976. Preparation and counts of particles in electron microscopy: application of negative stain in the agar filtration method. Microsc. Acta **78**:131–148.
63. **Kellenberger, E., and C. Kellenberger-van der Kamp.** An improved specimen preparation method for high power light microscopy: first results with in vivo fluorescently stained nucleoids and plasmolysis. J. Microsc., in press.
64. **Kellenberger, E., and A. Ryter.** 1964. In bacteriology, p. 335–393. *In* B. M. Siegel (ed.), Modern developments in electron microscopy. Academic Press, Inc., New York.
65. **Kellenberger, E., W. Schwab, and A. Ryter.** 1956. L'utilisation d'un copolymère du groupe des polyesters comme matériel d'inclusion en ultramicrotomie. Experientia **12**:421–422.

66. **Knaysi, G., and R. F. Baker.** 1947. Demonstration with the electron microscope of a nucleus in *Bacillus mycoides* grown in a nitrogen-free medium. J. Bacteriol. **53**:539–553.
67. **Knoell, H., and K. Zapf.** 1951. Untersuchungen zum Problem des Bakterienzellkerns. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. I Orig. **157**:389–406.
68. **Koch, R.** 1876. Die Aetiologie der Milzbrand-Krankheit begründet auf der Entwicklungsgeschichte des *Bacillus anthracis*. Beitr. Biol. Pflanzen **2**:277–308.
69. **Kuroiwa, T.** 1982. Mitochondrial nuclei. Int. Rev. Cytol. **75**:1–59.
70. **Livolant, F.** 1978. New observations on the twisted arrangement of dinoflagellate chromosomes. Chromosoma (Berlin) **68**:21–44.
71. **Løbner-Oleson, A., and P. L. Kuempel.** 1992. Chromosome partitioning in *Escherichia coli*. J. Bacteriol. **174**:7883–7889.
72. **Lutkenhaus.** 1993. FtsZ and bacterial cytokinesis. Mol. Microbiol. **9**:403–409.
73. **Mason, D. J., and D. M. Powelson.** 1956. Nuclear division as observed in live bacteria by a new technique. J. Bacteriol. **71**:474–479.
74. **McKerracher, L. M., and I. B. Heath.** 1985. Microtubules around migrating nuclei in conventionally-fixed and freeze-substituted cells. Protoplasma **125**:162–172.
75. **Migula, W.** 1897. System der Bakterien, Band 1, allgemeiner Teil. Gustav Fischer, Jena, Germany.
76. **Miller, O. L., B. A. Hamkolo, and C. A. Thomas, Jr.** 1970. Visualization of bacterial genes in action. Science **169**:393–394.
77. **Moncany, M. L. J.** 1982. Détermination des conditions intracellulaires chez *Escherichia coli*. Conséquences biologiques de leur modification. Thèse d'Etat, Universités de Bâle et Paris VII.
78. **Mukherjee, A., K. Dai, and J.-J. Lutkenhaus.** 1993. *Escherichia coli* cell division protein FtsZ is a guanine nucleotide binding protein. Proc. Natl. Acad. Sci. USA **90**:1053–1057.
79. **Nanninga, N., and C. L. Woldringh.** 1985. Cell growth, genome duplication and cell division, p. 259–318. *In* N. Nanninga (ed.), Molecular cytology of *Escherichia coli*. Academic Press, Ltd., London.
80. **Nash, H. A.** 1990. Bending and supercoiling of DNA at the attachment site of bacteriophage lambda. Trends Biochem. Sci. **15**:222–227.
81. **Neumann, F.** 1941. Untersuchungen zur Erforschung der Kernverhältnisse bei den Bakterien. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. I Orig. **103**:385–400.
82. **Okada, Y., M. Wachi, A. Hirata, K. Suzuki, K. Nagai, and M. Matsuhashi.** 1994. Cytoplasmic axial filaments in *Escherichia coli* cells: possible function in the mechanisms of chromosome segregation and cell division. J. Bacteriol. **176**:917–922.
83. **Olive, L. S.** 1953. The structure and behavior of fungal nuclei. Bot. Rev. **19**:439–586.
84. **Pettijohn, D. E.** 1982. Structure and properties of the bacterial nucleoid. Cell **30**:667–669.
85. **Pettijohn, D. E., and O. Pfenniger.** 1980. Supercoils in prokaryotic DNA restrained *in vivo*. Proc. Natl. Acad. Sci. USA **77**:1331–1335.
86. **Piéchaud, M.** 1954. La coloration sans hydrolyse du noyau des bacteries. Ann. Inst. Pasteur (Paris) **86**:787–793.
87. **Piekarski, G.** 1937. Zytologische Untersuchungen an Paratyphus- und Coli Bakterien. Arch. Mikrobiol. **8**:428–429.
88. **Raikov, I. B.** 1982. The protozoan nucleus: morphology and evolution. Springer-Verlag, Vienna.
89. **Rizzo, P. J.** 1981. Comparative aspects of basic chromatin protein in dinoflagellates. BioSystems **14**:433–443.
90. **Robinow, C. F.** 1942. A study of the nuclear apparatus of bacteria. Proc. R. Soc. London Ser. B **130**:299–324.
91. **Robinow, C. F.** 1944. Cytological observations on *Bact. coli*, *Proteus vulgaris* and various aerobic spore-forming bacteria with special reference to the nuclear structures. J. Hyg. **43**:413–423.
92. **Robinow, C. F.** 1945. Addendum: nuclear apparatus and cell structures of rod-shaped bacteria, p. 355–377. *In* R. Dubos, The bacterial cell in its relation to problems of virulence, immunity and chemotherapy. Harvard University Press, Cambridge, Mass.
93. **Robinow, C. F.** 1956. The chromatin bodies of bacteria. Bacteriol. Rev. **20**:207–242.
94. **Robinow, C. F.** 1957. The structure and behaviour of the nuclei in spores and growing hyphae of Mucorales. Can. J. Microbiol. **3**:771–789.
95. **Robinow, C. F.** 1960. Outline of the visible organization of bacteria, p. 45–108. *In* J. Brachet and A. E. Mirsky (ed.), The cell, vol. IV. Biochemistry, physiology, morphology. Academic Press, Inc., New York.
96. **Robinow, C. F.** 1962. Morphology of the bacterial nucleus. Br. Med. Bull. **18**:31–35.
97. **Robinow, C. F.** 1962–1963. Problems of nuclear behavior in bacteria and fungi, p. 1–18. *In* Lectures on theoretical and applied aspects of modern microbiology, vol. 2. University of Maryland, College Park.
98. **Robinow, C. F.** 1981. Nuclear behavior in conidial fungi, p. 357–393. *In* G. T. Cole and B. K. Kendrick (ed.), Biology of conidial fungi, vol. 2. Academic Press, Inc., New York.
99. **Robinow, C. F., and J. S. Hyams.** 1989. General cytology of fission yeasts, p. 273–330. *In* A. Nasim, P. Young, and B. F. Johnson (ed.), Molecular biology of the fission yeast. Academic Press, Inc., New York.
100. **Robinow, C. F., and B. F. Johnson.** 1991. Yeast cytology: an overview, p. 8–119. *In* A. H. Rose and J. H. Harrison (ed.), The yeasts, vol. 4. Academic Press, Ltd., London.
101. **Robinow, C. F., and R. G. E. Murray.** 1953. The differentiation of cell wall, cytoplasmic membrane and cytoplasm of Gram positive bacteria by selective staining. Exp. Cell Res. **4**:390–407.
102. **Roth, J., and N. Kleckner.** 1993. Bacterial chromosomes. J. Cell. Biochem. Suppl. **17E**:273–310.
103. **Ryter, A.** 1960. Etude au microscope électronique des transformations nucléaires de *Escherichia coli* K12S et K12 (lambda), après irradiation aux rayons ultraviolets et aux rayons X. J. Biophys. Biochem. Cytol. **8**:399–412.
104. **Ryter, A., and A. Chang.** 1975. Localization of transcribing genes in the bacterial cell by means of high resolution autoradiography. J. Mol. Biol. **98**:797–810.
105. **Ryter, A., and E. Kellenberger.** 1958. L'inclusion au polyester pour l'ultramicrotomie. J. Ultrastruct. Res. **2**:200–214.
106. **Ryter, A., E. Kellenberger, A. Birch-Andersen, and O. Maaløe.** 1958. Etude au microscope électronique des plasmas contenant de l'acide désoxyribonucléique. I. Les nucléoides des bactéries en croissance active. Z. Naturforsch. **13b**:597–605.
107. **Sabatini, D. D., K. Bensch, and R. J. Barrnett.** 1963. Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. J. Cell Biol. **17**:19–58.
108. **Schmid, M. B.** 1988. Structure and function of the bacterial chromosome. Trends Biochem. Sci. **13**:131–135.
109. **Schreil, W. H.** 1964. Studies on the fixation of artificial and bacterial DNA plasms for the electron microscopy of thin sections. J. Cell Biol. **22**:1–20.
110. **Séchaud, J., and E. Kellenberger.** 1972. Electron microscopy of DNA-containing plasms. IV. Glutaraldehyde-uranyl acetate fixation of virus-infected bacteria for thin sectioning. J. Ultrastruct. Res. **39**:598–607.
111. **Setlow, B., N. Magill, P. Febroriello, L. Nakhimovsky, D. E. Koppel, and P. Setlow.** 1991. Condensation of forespore nucleoid early in sporulation of *Bacillus* species. J. Bacteriol. **173**:6270–6278.
112. **Seward, M. R.** 1970. Viable spores recovered from an archaeological excavation. Nature (London) **261**:407–408.
113. **Sigee, D. C.** 1984. Structural DNA and genetically active DNA in dinoflagellate chromosomes. BioSystems **16**:203–210.
114. **Soyer, M.-O., and O. K. Haapala.** 1974. Structural changes of dinoflagellate chromosomes by pronase and ribonuclease. Chromosoma **47**:179–192.
115. **Spector, D. L.** 1984. Dinoflagellate nuclei, p. 107–147. *In* D. L. Spector (ed.), Dinoflagellates. Academic Press, Inc., Orlando, Fla.
116. **Stempen, H.** 1950. Demonstration of the chromatinic bodies of *Escherichia coli* and *Proteus vulgaris* with the aid of the phase contrast microscope. J. Bacteriol. **60**:81–100.
117. **Stille, B.** 1937. Zytologische Untersuchungen an Bakterien mit Hilfe der Feulgenschen Nuclealreaktion. Arch. Mikrobiol. **8**:124–148.

117a. **Taubeneck, U.** 1969. Virusproteine in Bakterienzellen. Z. Allg. Mikrobiol. **9**:315–326.
118. **Trun, N. J., and S. Gottesman.** 1990. On the bacterial cell cycle: E. coli mutants with altered ploidy. Genes Dev. **4**:2036–2047.
119. **Tulasne, R., and R. Vendrely.** 1947. Demonstration of bacterial nuclei with ribonuclease. Nature (London) **160**:225.
120. **van Iterson, W.** 1965. Symposium on the fine structure and replication of bacteria and their parts. II. Bacterial cytoplasm. Bacteriol. Rev. **29**:290–325.
121. **van Iterson, W.** 1984. Benchmark papers in microbiology, vol. 17. Inner structures of bacteria. Van Nostrand Reinhold Co., New York.
122. **van Iterson, W.** 1984. Benchmark papers in microbiology, vol. 18. Outer structures of bacteria. Van Nostrand Reinhold Co., New York.
123. **van Iterson, W., F. M. Hoeniger, and E. Nijman Van Zanten.** 1967. A "microtubule" in a bacterium. J. Cell Biol. **32**:1–10.
124. **van Iterson, W., and C. Robinow.** 1961. Observation with the electron microscope on the fine structure of nuclei of two spherical bacteria. J. Biophys. Biochem. Cytol. **9**:171–181.
125. **Vernet, G., M. Sala-Rovira, M. Maeder, F. Jacques, and M. Herzog.** 1990. Basic nuclear proteins of the histone-less eukaryote *Crypthecodinium cohnii* (Pyrrhophyta): two-dimensional electrophoresis and DNA-binding properties. Biochim. Biophys. Acta **1048**:281–289.
126. **Whitfield, J. F., and R. G. E. Murray.** 1956. The effects of the ionic environment on the chromatin structures of bacteria. Can. J. Microbiol. **2**:245–260.
127. **Williamson, D. H., and D. J. Fennel.** 1975. The use of fluorescent DNA-binding agents for detecting and separating yeast mitochondrial DNA. Methods Cell Biol. **12**:335–351.
128. **Woldringh, C. L., and N. Nanninga.** 1985. Structure of nucleoid and cytoplasm in the intact cell, p. 161–197. *In* N. Nanninga (ed.), Molecular cytology of *Escherichia coli*. Academic Press, Ltd., London.